

**SINGLE DOSE KILLING PHARMACODYNAMICS  
OF ANTIMALARIAL DRUGS IN A LUCIFERASE  
MURINE MALARIA MODEL**

by  
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# ABSTRACT

Malaria is one of the leading causes of high infant morbidity & mortality in children aged five years and below in the sub-Saharan Africa. Emergence of resistance to all classes of antimalarial drugs is currently a huge challenge for the fight against malaria. Combination therapies of rapid and slow killing antimalarial drugs is the current first-line therapy to achieve complete parasite clearance or cure. The majority of previous laboratory-based malaria preclinical drug studies have relied on drug inhibition of low numbers of parasites numerically compared to untreated controls. In contrast, human malaria drug studies measure the decline or killing in high numbers of parasites numbering near a trillion per person. We therefore hypothesize that single dose or combination pharmacodynamics of *Plasmodium* drug killing in a high parasitemia *in vivo* mouse malaria model is different from parasite *P. falciparum* inhibition *in vitro*.

We assessed the *in vivo* single dose dynamic killing pharmacodynamics properties of artesunate and 4-aminoquinolines (pyronaridine, chloroquine, and amodiaquine). Parasiticidal metrics employed were parasite log reduction, undetectable parasitemia duration, speed & duration to recrudescence (initial parasitemia) and 30-day cure in a *Pberghei*ANK4-GFP-Luciferase-based murine malaria blood stage model that has an untreated parasite multiplication rate of a 9 fold increase over 24 hours. Our model waits 4-days post infection for the 3-5% high parasitemia so that one can measure parasite clearance or log reduction.

In this study, pyronaridine exhibited dose-independent killing, achieving parasite reduction near 5-6 logs at 48 hours post dosing with complete cure at 10 mg/kg compared to artesunate, which exhibited a 48 hour dose-dependent killing with a 2 log drop at the noncurative high dose of 250 mg/kg. Chloroquine (noncurative) and amodiaquine (partially curative) have nearly the same initial dose-independent killing with a lag phase of no parasite reduction between 6 and 24 hours post dosing, but an 2.5 log reduction at 48 hours. In drug treated, washed infected blood transfer experiments to a naïve mouse, chloroquine and amodiaquine showed less viable parasites at the 24

hour compared to 8 hour transfer measured by a prolonged return to parasitemia, despite a similar log reduction, in contrast to the correlation of parasite log reduction to viable parasites with artesunate and pyronaridine. Artesunate in combination with pyronaridine exhibited a slightly weak antagonistic effect, while the combination with chloroquine or amodiaquine, showed an additive effect. Single oral dose pyronaridine was much more potent *in vivo* than artesunate, chloroquine and amodiaquine.

## THESIS READERS

Dr. David Sullivan, Molecular Microbiology & Immunology (JHSPH) – Advisor, Primary Reader,

Prof. Clive Shiff, Molecular Microbiology & Immunology (JHSPH) – Secondary Reader

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entire support system who continuously push me to be the best, continue shooting for the stars as I follow and lead my dreams, such a huge blessing and I'm so grateful to God. To whom much is given much is expected. I come from humbling backgrounds and Western Kenya area that is mostly affected by malaria disease among other infectious diseases. In my country, there are very few research and capacity building infrastructures at the moment. Investing in the education and training of the next generation of young African scientists and public health professionals will ease and hasten the fight against the infectious diseases such as malaria that are endemic in Africa. I look forward to pursuing my PhD studies and eventually envision taking my knowledge & skills and returning to Kenya, Africa to help make a difference in public health field as a change agent and help nurture and empower young upcoming scientists and public health professionals. One of my biggest dreams is to help build and run a top-notch state-of-the-art school of public health and research institute in Kenya through collaborative efforts. I have faith this dream will one day come true.

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# **CHAPTER 1 - INTRODUCTION**

## **GLOBAL BURDEN OF INFECTIOUS TROPICAL DISEASES**

Infectious diseases occur and thrive in the tropical and subtropical regions such as the sub-Saharan Africa and south-east Asia where the environmental conditions are humid and hot. Some of the tropical infectious diseases include: neglected tropical infectious diseases (NTDs), HIV/AIDS, tuberculosis and malaria. First, the NTDs, are communicable diseases that impact and predominate in the low-income poverty stricken & underprivileged regions with poor hygiene and sanitation measures in the tropical regions. According to WHO, NTDs prevail in 149 countries affecting more than a billion people globally and low-income countries are affected by at least five NTDs. This subset of tropical diseases has been neglected by researchers, policy-makers. NTDs are a subset of 17 bacterial, protozoal, parasitic and viral infections including: human African trypanosomiasis (sleeping sickness), chagas disease, dengue & chikungunya, guinea-worm disease, echinococcosis, buruli ulcer, leishmaniasis, leprosy, lymphatic filariasis, rabies, food-borne trematodiasis, schistosomiasis, soil-transmitted helminthiasis, trachoma, yaws, taeniasis and onchocerciasis. (Feasey N et al., 2010; Bhutta et al., 2014; Dye C 2014; Hotez PJ et al., 2004).

Second, HIV & AIDS. Human immunodeficiency virus (HIV) can lead to disease known as the acquired immunodeficiency syndrome (AIDS) if left untreated. HIV virus attacks human host's immune system, specifically the immune T cells known as CD4 cells that fight off infection, and drastically lowers the number of these immune cells hence weakening the host's immune system making the victim vulnerable to many other opportunistic infections. AIDS is considered the worst stage of HIV infection when the host's immune cells CD4 counts have dropped below 200 cells per cubic millimeter of blood, and/or they have acquired other opportunistic infections. HIV/AIDS is considered a sexually transmitted disease that can also be transmitted through infected body fluids such as blood, breast milk, and by sharing contaminated needles. HIV/AIDS is one of the greatest killers and public health challenge in the low and middle income countries (LMIC) around the World. Since the emergence of AIDs in 1981, more than 30 million infected with HIV have died. According

to UNAIDS global HIV statistics: by the end of 2015 nearly 36.7 million people globally were living with HIV and of these 1.8 million were children below 15 years old, 1.1 million people died from AIDS-related illnesses, and worldwide new HIV infections was at 2.1 million people. As of mid-2016, approximately 18.2 million people are receiving antiretroviral treatment globally. (UNAIDS 2016; Bhutta ZA et al., 2014).

Third, Tuberculosis (TB) is the second greatest killer in part due to HIV/AIDS infections, and one of the top 10 causes of death worldwide. Nearly 95% of TB deaths occur in the LMIC with highest burden in Asia and Africa. According to CDC and WHO reports, approximately one third of the world population is infected with TB. Of the estimated 2-3 billion people infected with TB only about 5-15% develop TB disease in their lifetime. Reports have shown that in 2015 nearly 10.4 million people were infected with TB while 1.8 million others died from the disease. The 60% of all the TB-related deaths were mainly in Asia and African countries (South Africa, Indonesia, China, Nigeria, Pakistan, and China). [WHO]. TB is also the leading killer of the HIV patients and in 2015 it accounted for 35% of HIV deaths. However, due to the effective use of the antiretroviral therapy, the number of TB-related deaths among HIV patients fell by 32% since 2004. [UNAIDS 2016]. Interestingly, in 2015 the mortality rate was reported to be 47% lower than in the early 90's and currently there are about 22 high TB burden countries accounting for the 80% TB cases worldwide. (UNAIDS 2016). Tuberculosis is an infectious airborne disease caused by the bacilli *Mycobacterium tuberculosis* (Mtb), a gram negative facultative intracellular bacteria that colonizes the lungs and airways, and can grow and replicate inside macrophages. Transmission of TB occurs via respiratory route when a person inhales and gets exposed to Mtb bacilli via airborne droplets exhaled through cough or sneeze from TB infectious individual. The inhaled bacilli deposit in the alveoli – air sacs in the lungs where they enter and multiply inside the alveolar macrophages. However, some of the bacilli escape the immune response and spread by the lymphatic or bloodstream channels to the rest of the lungs and other distant body tissues and organs. This rapid dissemination prime's the host's immune system for massive or "systemic" immune response to combat the mycobacteria. There are

two kinds of TB: primary tuberculosis disease and Latent TB infection (LTBI). About one third of the world population has the latent tuberculosis. TB is both preventable, treatable and curable disease using the antimicrobial drugs majorly the isoniazid and rifampicin, however emergence of anti-TB drug resistance is one of the challenges facing fight against TB and has led to another form of TB known as the multidrug-resistant tuberculosis (MDR-TB) (Korb VC et al., 2016).

Fourth, Malaria is also a major public health challenge and the great single tropical infectious disease killer of all time that continues to rob many lives, especially pregnant women and children under the age of five years old in sub-Saharan Africa and South-East Asia. Malaria has killed more than 30 million people since 1981 which was the start of the AIDS epidemic. The global burden of malaria disease is discussed to a greater in depth in the next chapters.

# GLOBAL BURDEN OF MALARIA

## Malaria History Overview

Malaria has been the most ubiquitous infectious disease known for many centuries as a public health threat worldwide. Malaria has afflicted human race throughout the evolutionary age and history dating back to the ancient Egyptians and Greeks around B.C. Table (i) shared below briefly outlines the malaria history timeline and highlights some of the key malaria discoveries and events from pathogenesis, diagnosis, chemoprevention, vaccine, preventive measures as well as challenges experienced in fight against malaria such as development of drug resistance.

**Table (i): Malaria History Timeline**

Timeline	Key Discoveries & Events
<b>30 million years' old</b>	Fossils of mosquitoes shows malaria vector present before the earliest history
<b>2700 BCE</b>	<ul style="list-style-type: none"><li>• Malaria symptoms (periodic fevers) described in ancient Chinese medical writings.</li><li>• Malaria became widely recognized in Greece by the 4th century BCE. Hippocrates noted the principal symptoms.</li><li>• During 2nd century of BCE, in China the Qinghao plant (<i>Artemisia annua</i>) was described in medical treatise.</li></ul>
<b>284-363 BCE</b>	Ge Hong of East Yin Dynasty first describes anti-fever properties of and recommends Qinghao ( <i>Artemisia annua</i> ) effusion for alleviating malaria symptoms
<b>1631</b>	Description of Quinine ( <i>Chinchona</i> bark) in Peru and transport to Rome for malaria therapy.
<b>1820</b>	Quinine was first purified from tree bark, which had been used to treat malaria for many years' prior
<b>1874</b>	German chemistry student, Othmer Zeidler, synthesized DDT for his thesis
<b>1880</b>	Charles Louis Alphonse Laveran first identifies the malaria parasite. (He was awarded Nobel Prize in 1907 for the discovery).
<b>1886</b>	Differentiation of species of malaria by Italian neurophysiologist known as Camillo golgi. He received Nobel Prize in 1906 for his work.
<b>1890 - 1931</b>	<p>Naming of human malaria parasites.</p> <ul style="list-style-type: none"><li>• In 1890 the names <i>Plasmodium vivax</i> and <i>Plasmodium malariae</i> were first introduced by Italian investigators Giovanni Batista Grassi and Raimondo Filetti.</li><li>• Alphonse Laveran, who was the first to observe parasites in a malaria patient's blood, believed there was only one species known as <i>Oscillaria malariae</i></li></ul>



	<ul style="list-style-type: none"> <li>• In 1897 an American, William H. Welch reviewed the naming of human malaria parasites and he named the malignant tertian malaria parasite <i>P.falciparum</i></li> <li>• In 1922, John William Watson Stephens described the fourth human malaria parasites <i>P.ovale</i></li> <li>• In 1931, Robert Knowles and Biraj Mohan Das Gupta first described <i>P.knowlesi</i>. however the first documented infection with <i>P.knowlesi</i> was in 1965.</li> </ul>
<b>1891</b>	Guttmann and Ehrlich describe the use of methylene blue against malaria, launching efforts for synthetic antimalarials and path eventually leading to Chloroquine.
<b>1897-1898</b>	Discovery by Sir Ronald Ross that mosquitoes transmit malaria (He was awarded Nobel Prize in 1902 for his work).
<b>1898-1899</b>	Discovery the transmission of human malaria parasites <i>Plasmodium</i> by Italian investigators led by Giovanni Batista Grassi.
<b>1934</b>	Hans Andersag discover the antimalarial drug known as Chloroquine (Resochin) in Germany, which was only widely used after World War II.
<b>1939</b>	Paul Hermann Muller tests the insecticide Dichloro-diphenyl-trichloroethane (DDT) in Switzerland. (He was awarded Nobel Prize in 1948).
<b>1946</b>	<ul style="list-style-type: none"> <li>• Primaquine, only marketed anti-relapse agent for P.vivax, first synthesized in the USA.</li> <li>• Chloroquine finally recognized and established as an effective and safe antimalarial by British and USA scientists.</li> </ul>
<b>1947</b>	Commercialization and widespread use of Chloroquine
<b>1951</b>	Malaria disease is eliminated in the United States
<b>1955</b>	WHO launches Global Malaria Eradication Campaign, excluding sub-Saharan Africa.
<b>1957</b>	First documented case of resistance to Chloroquine reported. Late 1950's Chloroquine resistance emerges on Thai-Cambodian border.
<b>1969</b>	The malaria eradication efforts are halted.
<b>1970's</b>	Chloroquine resistance documented on every continent with malaria
<b>1972</b>	Artemisinin was isolated from <i>Artemisia annua</i> by Chinese chemist, Tu Youyou. ( <i>Derivatives of this extract are today's most potent and effective antimalarial drugs.</i> )
<b>1976</b>	William Trager and JB Jensen grow malaria parasite in culture for the first time. This opened door for drug discovery and vaccine development.
<b>Late 1970's</b>	Resistance to sulfadoxine-pyrimethamine (SP) emerges
<b>1989</b>	The FDA approves the use of the antimalarial drug Mefloquine hydrochloride, brand name known as Lariam® by Hoffmann-LaRoche
<b>1992</b>	Malaria vaccine candidate RTS,S is developed by GlaxoSmithKline and the Walter Reed Army Institute. Clinical trials started the same year.
<b>Mid 1990's</b>	Sulfadoxine-pyrimethamine resistance documented on every continent with malaria.

<b>1996</b>	Insecticide-treated bednets proven to reduce overall childhood mortality by 20% in large in multi-country African study.
<b>1998</b>	<ul style="list-style-type: none"> <li>• Roll Back Malaria partnership launched by WHO, UNICEF, UNDP and World Bank. Key goal: halving malaria incidence and mortality by 2010.</li> <li>• WHO adopts home management strategy for malaria; trained community volunteer health workers provide antimalarials in remote African communities.</li> </ul>
<b>Late 1990's</b>	Mefloquine resistance emerges in the Southeast Asia
<b>2000</b>	UN general assembly adopts the Millennium Development Goals (MDGs) and set target to halt and begin reversing malaria incidence by 2015.
<b>2001</b>	WHO prequalifies first fixed-dose Artemisinin combination therapy (ACT) known as Coartem® (Artemether/lumefantrine) for treatment of uncomplicated malaria caused by <i>P.falciparum</i> . ACT is recommended as first-line malaria treatment.
<b>2002</b>	<ul style="list-style-type: none"> <li>• The Global Fund to Fight AIDS, TB, and Malaria is established, led by UCSF's Sir Richard Feachem.</li> <li>• Genome sequencing of malaria vector (<i>Anopheles gambiae</i>) and parasite (<i>Plasmodium falciparum</i>) is completed.</li> </ul>
<b>2005</b>	World health assembly adopts 80% worldwide coverage of insecticide nets and ACTs by 2010.
<b>2010</b>	Prequalification of Artesunate injections for severe malaria by Guilin Pharmaceuticals
<b>2009 - 2012</b>	Launch of five fixed-dose ACTs by MMV, DNDi and pharmaceutical partners

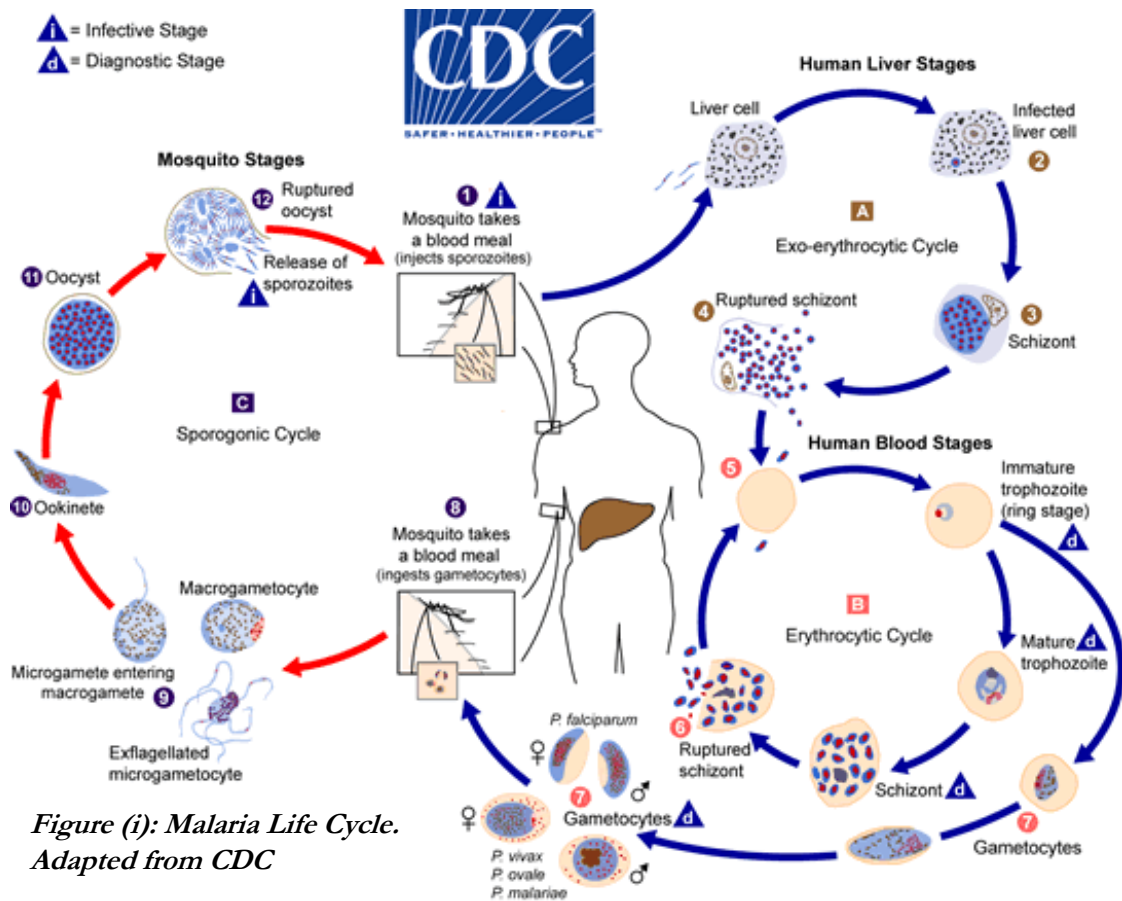
Reference: CDC 2016, Nobel Prize 2016, Miller RL et al 1994, UCSF 2011, Wells TNC et al 2015

# **MALARIA EPIDEMIOLOGY**

## **Morbidity & Mortality**

Malaria is a severe life-threatening killer disease in the tropical & subtropical regions especially in the sub-Saharan Africa where there is still huge burden of the disease. Estimated malaria mortality rates are highest in the endemic countries with lower gross national income per capita and nearly half of the world population is at risk of malaria. According to the 2016 world malaria report there were 212 million new cases of malaria worldwide in 2015, with African region accounting for almost 90% of the global malaria cases, followed by Southeast Asia region (7%) and the Eastern Mediterranean region (2%). The 2016 report further documents that there were approximately 429,000 malaria deaths worldwide, with most of the deaths (92%) occurred in African region, 6% in South-East Asia region and 2% in Eastern Mediterranean region. Between the years 2010 and 2015, malaria incidence rates fell by 21% in African region and globally. In areas with high transmission, pregnant women and children below 5 years old are the most vulnerable groups and at high risk of malaria disease in the endemic populations; more than 70% of all malaria deaths occur in the 5 years and under age group, particularly susceptible to malaria morbidity, infection and death. In 2015, the disease killed approximately 303,000 children under five years of age globally. The greatest percentage-96% was from the African region (292,000 deaths). However, the report affirms that malaria mortality rate among children 5 years and below fell by 35% and overall, the malaria mortality rates also fell by nearly 31% in African region and by 29% globally between 2010 and 2015 among all age groups. (WHO-World Malaria Report 2016).

## MALARIA DISEASE LIFE CYCLE & TRANSMISSION



**Figure (i): Malaria Life Cycle.**  
 Adapted from CDC

Malaria is a life-threatening killer disease that is caused by the *Plasmodium* species parasites transmitted by the infected *Anopheles spp.* female mosquito vectors when they blood feed on human host. Human malaria is caused by *P. falciparum* and four other *Plasmodium* species that have also been recognized to be infect humans and these include *P. malariae*, *P. vivax*, *P. ovale*, and *P. knowlesi*. The human malaria life cycle involves two hosts: mosquito vector and human host. The human cycle entails the exo-erythrocytic cycle in the liver and the erythrocytic stage in the blood. One of the key bottleneck stages is the sporozoites infecting the liver. When the infected female mosquito vector takes blood meal it injects sporozoites, which then invade the hepatocytes (liver cells) and become invasive and slow down their movement speed. The majority of the sporozoites deposited do not reach the blood vessels, some die in the skin, while others are taken up by the antigen presenting cells

such as macrophages and others get into the drainage lymph nodes. Up to 10% of sporozoites transform into the merozoite stage in the liver and it is estimated that one sporozoite results into about 5000 to 10,000 hepatic merozoites. (Ijeoma and Sinnis 2009). Liver merozoites initiate the asexual blood stage (erythrocytic) by invading the erythrocytes (RBCs) forming ring stage – asexual haploid cell division, trophozoite, schizonts, merozoites, (another cycle of ring stage), and then gametocytes. The ring stage is only seen evidently in the peripheral circulation with the *P. falciparum* because this species is capable of infecting all RBC ages (both young and old), and the trophozoites cytoadhere on the endothelial cells via surface antigens to evade splenic clearance.

The sexual stage takes place in the mosquito, also known as sporogonic cycle, begins when the female *Anopheles* mosquito blood feeds on an infected human host and ingests the *Plasmodium* gametocytes which are at a relatively low density in the blood meal compared to the density of asexuals in the human host. Then gametocytosis begins- macrogametes and microgametes fuse to form a zygote. The zygote develops into a motile ookinete that moves to the mosquito midgut and develops into oocysts, which is the other key bottleneck stage: the number of oocysts formed. The oocysts release sporozoites into the hemocoel, which traverse into the salivary gland.

When the mosquito takes another blood meal, it injects the sporozoites from the salivary gland into the human skin. During blood meal, sporozoites are released from the salivary glands as mosquito probes and salivates in the human host and only stops when she finds blood. In the mosquito oocyst, the sporozoites have the highest insect density of *Plasmodium* parasites followed by the salivary gland sporozoites, with the lowest being those delivered into the human skin, where only 1% reach the blood stream enroute to the liver. For the human host cell invasion, the *Plasmodium* parasite (sporozoites) uses gliding motility and cell traversal mechanisms during which the human host cell becomes wounded. (Ijeoma and Sinnis 2009).

## **MALARIA DIAGNOSIS AND PATHOGENESIS**

Malaria disease diagnosis methods that have been employed thus far include clinical diagnosis and blood examination. Clinical diagnosis looking at key symptoms such as fever, splenomegaly and anemia are still being used in malaria endemic countries in sub-Saharan Africa but grossly over-estimates the infection. The blood examination diagnosis methods include the use of microscopy (blood films examination using Giemsa staining to detect malaria parasites), Rapid Diagnostic Tests (RDT) used for aldolase, lactate dehydrogenase and histidine-rich protein-2 (Hrp-2) detection, DNA – genomic detection using real-time PCR or nested PCR, RNA detection of asexual blood stages and gametocytes using reverse transcriptase real-time PCR, and using serology or ELISA for antibody detection. Microscopy use of blood films is considered the gold standard diagnosis tool however it has issues such as being very hard to visualize or distinguish specific parasite blood stages, it is time consuming and requires intensive training and practice. (WHO 2016 – Diagnostic Testing).

Malaria diagnosis is both density and volume dependent. An RDT samples in the range of 10 to 20 microliters of blood more than the 0.1 microliter sampled by microscopy even though both have nearly same sensitivity. Most of the RDTs accommodate maximum volume of up to 20 microliters of whole blood , however it is not a quantitate test but rather gives a yes/no response on the presence of the *P. falciparum*. Also the malaria RDT cassettes come in different sizes and this affects the efficiency of the test. RDT is one of the current measures of malaria diagnosis that is much faster (provide rapid results within 15-30 minutes, are relatively simple to perform and interpret, and require very limited training on how to use) than microscopy which majorly relies on microscopes, well-trained technicians and the necessary reagents that are scarce resources in the malaria endemic areas. Rapid Diagnostic Testing (RDT) is readily available for use in malaria epidemics and emergencies though one of the problems facing this diagnosis test is the high asymptomatic parasitemia cases in most of the sub-Saharan Africa. However, the downside of RDT is that it is expensive- one test can cost up to \$2.50, many RDT only target HRP-II for detection and

therefore only suitable for *P. falciparum*. WHO highly recommends use of RDTs for malaria diagnosis before initiating treatment. (WHO 2016, JHSPH – MMI 2015 Malariology Lecture on diagnosis).

## **Classic Clinical Human Malaria**

This is the most lethal and killer version of the disease and it is associated with the asexual blood stage parasite cycle in the human host. The malarial parasite releases various toxic components inside the erythrocyte such as hemozoin (malaria pigment) and parasite proteins upon schizont rupture. These components exacerbate the disease by triggering the host's immune cells (macrophages and other innate antigen presenting cells) to illicit symptoms such as fever, one of the clinical manifestation of malaria disease. The other symptoms are anemia due to hemolysis and enlarged spleen (splenomegaly), severe cases manifest severe anemia and cerebral coma. *P. falciparum* causes more lethal clinical disease because it infects both young and old RBCs, it has high amplification, causes end organ failure, and it undergoes through the process of sequestration. *P. falciparum* has the surface proteins (polymorphism and antigenic variation to evade the host immune response) such as the erythrocyte membrane protein PfEMP-1 expressed on the surface of the infected red blood cell and it therefore cytoadheres on the surface of host receptors expressed on the endothelial cells. The PfEMP-1 ligand accounts for the sequestration hence only the ring-stage of the parasite is detected within the circulation (periphery) and the mature stages are sequestered in the blood vessels such as the capillaries and other organs. (JHSPH – MMI 2015 Malariology Lecture on diagnosis).

## **Asymptomatic Malaria**

It exists largely in the malaria endemic areas especially among the children and adult persons who have had repeated exposure to the disease. Mostly the asymptomatic malaria cases are untreated and clinically undetectable since people don't feel sick or manifest the clinical symptoms as much, therefore most of the asymptomatic people don't visit health clinics or seek medical attention. Asymptomatic cases therefore serve as reservoirs for the malaria parasite's gametocytes that infect

the *Anopheles* mosquitoes during blood meals and this extends the parasite's life cycle when a new person is bitten by the infected vector mosquito. Asymptomatic malaria contributes largely to transmission and it can be detected using other measures such as doing blood surveys and slide positivity cross-sectional test within a community of both adults and young people to determine parasite prevalence. (Laishram DD et al., 2012; Snow RW et al., 2012; Chen I et al., 2016).

## **Malaria Pathogenesis**

Malaria pathogenesis risk factors include mosquito vector, human host, the malarial parasites, and the ecological or environmental factors. Mosquito factors include the vector competence – the measure of the vector's ability to be infected by and transmit the *Plasmodium* parasite. Therefore, the higher the vector competence and density (vectorial capacity) the higher the transmission. The human host factors include immunity response, genetics and age. Humans have developed selective factors such as genetic polymorphism like the sickle cell trait whereby the red blood cells acquire a sickle-shape that protects against *P. falciparum* malaria disease however this manifests into a serious disease, sickle cell anemia, in most of the malaria endemic areas in Africa. The host immune response antibodies are effective in fighting the sporozoites by binding on the circumsporozoite protein. Antibody protection inhibits mobility of parasite into skin-liver-bloodstream, however it requires very high levels of antibody, high binding affinity and efficient immunological memory. Development of anti-parasite immunity in the malaria endemic areas where most adults and children are asymptomatic and serve as reservoirs, however infants and children five years and below have weaker immunity and therefore first couple of exposures to malarial parasite results into severe cases such as cerebral malaria, severe anemia, as well as death.

Cerebral malaria is a case scenario of when the host immune system over-reacts (cytokine storm) to the infection and this worsens the disease. Some of the parasite factors include: species, strain and density. In the severe malaria cases most parasites are sequestered and cytoadhere on the endothelial cells. The *Plasmodium* species have differences and *P. falciparum* is the most virulent and invades both young and old RBCs, there is sequestration of the infected RBC and severe case results



into cerebral malaria, severe anemia. The parasite also has different strain differences and diverse surface antigens that undergo antigenic polymorphism to evade the host's immune system.

Environmental factors affecting transmission include space and time, different types of rainfall can influence the level of pathology and transmission.

## **MALARIA CHEMOPREVENTION – ANTIMALARIAL DRUGS**

In malaria endemic areas some of the prevention measures that have been taken to reduce malaria transmission include using indoor residual spraying, insecticide impregnated bed nets, and application of repellants. However, along with all these measures malaria chemotherapy and prophylaxis is also essential. The key goal of treating uncomplicated and severe malaria disease is to prevent development of severe conditions reduce death rate and most importantly cure the disease. Currently chemotherapy of malaria depends on several drugs. Effectiveness of an antimalarial depends not only on the interactions between the drug and *Plasmodium* parasite but also on the drug and the host, more specifically on the pharmacokinetics and pharmacodynamics properties of drugs.

Antimalarial agents are classified as: blood schizonticides that act on the asexual erythrocytic stages of the malaria parasite, tissue schizonticides that inhibit liver stages, hypnozoitocides preventing relapses in *P. vivax* and *P. ovale* malaria and gametocytocides mainly target the sexual stage gametocytes to prevent transmission from human to mosquito. In *falciparum* malaria, the blood schizonticides are considered sufficient to cure the infection. The currently well-known classes of antimalarials include artemisinin & derivatives, quinolines (4-aminoquinolines – chloroquine, amodiaquine, and new drug of interest pyronaridine, 8-aminoquinolines such as primaquine and new drug tafenoquine), antifolates (proguanil, sulfadoxine, pyrimethamine) and antibacterials (atovaquone, doxycycline). (WHO).

### **Artemisinins & Derivatives**

Artemisinin and its derivatives are currently considered the most potent antimalarial agents and they have been efficacious in both uncomplicated and severe *falciparum* malaria. Besides artemisinin, the other derivatives developed include artesunate, artemether, dihydroartemisinin, arteether, which are currently considered the most effective antimalaria drugs to treat against human *falciparum* malaria. Artemisinin was discovered in China by Dr. Tu Youyou, who won the Nobel Prize in 2015 for this discovery. Artemisinin also known as qinghaosu, was isolated in 1972 from

sweet wormwood plant (*Artemisia annua*), where the drug is present in the leaves and flowers of this plant. The artemisinin structure was elucidated in 1979 and this enabled the development of derivatives. (Balint GA. 2001; Meshnick SR. 2002; Golenser J et al., 2006).

Artemisinin and its derivatives are endoperoxide-containing products and schizonticidal gametocytocidal drugs that targets the infected red blood cells from late ring to trophozoites and also gametocyte stages, destroying the sexual erythrocyte stages of the *Plasmodium* parasite and preventing further transmission to the mosquito during blood meal. Studies both preclinical and clinical have shown that artemisinin and derivatives are potent anti-malarial drugs that are effective against malaria parasites and multi-resistant strains (Li Q and Weina P. 2010) and achieve faster parasite clearance, even though they are short-lived. Artemisinin compound's mechanism of action involves activation by the heme iron or free iron which generates radical damage of nearby proteins, lipids and enzymes. Other observations on artemisinin action are lack of inhibition for heme crystallization, inhibition of the calcium ion transporter PfATPase6, interaction with the malaria protein Histidine-rich protein, increase in lipid damage and generation of carbon centered radicals without damage by oxygen radicals (Meshnick, SR 1998; Li Q et al., 2007).

Some of the pharmacological properties of artemisinin and derivatives are as follows: they have shorter biological or elimination half-life than aminoquinolines and the peak plasma concentrations are reached in about 1 to 2 hours. Of the derivatives, artesunate acts as the prodrug since it is rapidly converted into its active metabolite dihydroartemisinin (DHA) that accounts for artesunate's antimalarial activity. Artesunate's pharmacokinetic properties make it the most effective artemisinin compound currently used in ACTs for the oral treatment of uncomplicated malaria. (Lee IS, Hufford CD 1990).

The artemisinin & derivatives' route of administration varies, some are administered orally, intravenously or intramuscularly. Oral formulations are absorbed rapidly but incompletely. Artesunate can be administered through all routes and it achieves therapeutic plasma concentration

rapidly in either route of administration in contrast to other artemisinin derivatives. Studies have shown that artesunate administered intravenously is more effective for the treatment of both severe and complicated malaria and have reported that injectable administration of artesunate results in more systemic availability than the other artemisinin derivatives. (Li Q et al., 2007, de Vries & Dien 1996). For the treatment of severe malaria, currently WHO recommends use of the injectable intravenous or intramuscular artesunate for at least twenty-four hours followed by complete 3-day course treatment of Artemisinin Combination Therapies (ACTs). For adults and children above 6 months old, WHO recommends oral administration dose regimen of 5 mg/kg artesunate orally first day followed by 2.5 mg/kg second and third days in combination with mefloquine 15 mg/kg on the third day. WHO recommends the following dose regimen for the parenteral administration after dissolving the artesunate powder in 5% sodium bicarbonate: loading dose of 2 mg/kg followed by 1 mg/kg after 4 hours and 24 hours (total of 4 mg/kg). The currently recommended total therapeutic dose regimen range of artesunate-amodiaquine combination is 4 mg/kg body weight per day artesunate and 10 mg/kg body weight per dose amodiaquine free base. (WHO guidelines for treatment of malaria 3<sup>rd</sup> Edition). No major clinical toxicity manifestations have been seen in people except extreme cases such as neurotoxicity in murine malaria models such as mice. Adverse effects are typically mild with artemisinins including headache, dizziness, and weakness in adults, rare hypersensitivity reactions. (China Research Group 1982, Brewer TG et al. 1994).

One of the key drawbacks of artemisinin compound therapy besides recrudescence and being costly, is the emergence of delayed clearance or resistance to artemisinins. Artemisinin delayed clearance in *P. falciparum* first emerged and was reported in western Cambodia characterized by slow or delayed parasite clearance – reflecting reduced susceptibility of the ring stage parasites. Both *in vitro* and *in vivo* studies have identified molecular markers of artemisinin resistance and reported that mutations in the Kelch 13 (k13)-propeller domain are associated with the delayed parasite clearance. (Ashley EA et al., 2014; Dondorp AM et al., 2009; Noedl H et al., 2008).

## 4-Aminoquinolines

The 4-aminoquinolines belong to the overall quinoline class of antimalarial drugs and these include chloroquine, amodiaquine and pyronaridine. 4-aminoquinolines were the first synthetic class of the quinoline antimalarials to be used in treatment of malaria and chloroquine became the key agent of malaria therapy for at least four decades and most successful in clinical use until today due to resistance problems. During the World-War II period, chloroquine emerged as one of the active and least toxic quinoline derivatives given as 150 mg free base (60%) or 250 mg/kg tablet. (Hoekenga et al., 1952; O'Neil PM et al., 1998). It has been reported that chloroquine acts by interrupting hemozoin detoxification in the *Plasmodium* parasite as it grows inside the human host's RBC (Chou AC et al., 1980, 1998; Sullivan et al., 1996; Dorn A et al., Welles & Plowe 2001).

## Chloroquine

Chloroquine was discovered as alternative against quinine, which was considered malaria wonder drug for quite a long time, for both treatment and prophylaxis of malaria. However it was heavily and widely used south-east Asia and sub-Saharan Africa leading to chloroquine resistance problems in both *P. falciparum* malaria endemic regions. Chloroquine resistance was first detected in the Cambodia-Thailand Southeast Asia and Colombia South America in the 1950s before worldwide spread. In Africa, resistance was first detected in Kenya and Tanzania in 1970s, even though there was still quinine use at the moment, still malaria morbidity and mortality spiked up especially among children. In falciparum malaria, chloroquine resistance is linked to multiple mutations in PfCRT protein that functions as a transporter of unknown function in the parasite's digestive vacuole membrane. (Welles & Plowe 2001). Even though resistance to chloroquine reduced its clinical use, it is still used in special patient groups such as children and pregnant women, for treatment of nonfalciparum malaria and as a comparator for *in vitro* and *in vivo* preclinical testing of new antimalarial agents on properties such as pharmacokinetics and pharmacodynamics. (Moore BR et al., 2001).

According to WHO, chloroquine is a 4-aminoquinoline that has rapid schizontocidal activity against blood stages of *P. ovale*, *P. malariae* and against susceptible strains of *P. vivax* and *P. falciparum*. Chloroquine is currently used for acute malarial attacks – *P. malariae* and *P. falciparum* susceptible infections – and for prophylaxis for pregnant women and non-immune persons at risk, however, its use is prohibited in the chloroquine-resistant malaria regions. The recommended dose regimen by the WHO, (all dosage are described in terms of free base) and as follows: oral dose administered to adults, children and pregnant women given at 10 mg/kg on the first day then 5 mg/kg six to 8 hours later, and 5 mg/kg on day 2 and 3 in a single dose (total dose of 25 mg/kg). The parenteral administration is only considered in no resistance situations or if the patient is unable to take drug orally. According to CDC malaria treatment information: uncomplicated falciparum malaria acquired in areas without chloroquine resistance, a dose regimen of chloroquine 600 mg base (1000 mg salt) is to be given initially followed by 300 mg base (500 mg/kg salt) at 6, 24 and 48 hours post initial dose (total of 1500 mg base ~ 2500 mg salt). For prophylaxis in adults and pregnant women give 300 mg base weekly and children 5 mg/kg weekly. Absorption is efficient following oral administration and peak plasma concentration occur within 2 to 3 hours and it can be detected in the plasma and urine for up to 2 months and 4 months respectively upon single dose administration. Adverse effects are rare except for rare hypersensitivity and minor headaches based on the dose regimen taken or recommended. (CDC 2016, WHO 2016).

## Amodiaquine

Amodiaquine is another 4-aminoquinoline that is structurally related to and has mode of action like chloroquine and it is also active against asexual *Plasmodium* stages. Amodiaquine was synthesized in 1946 and first introduced as an alternative antimalarial due to its effective activity against the chloroquine-resistant falciparum parasites. However, currently highly chloroquine-resistant falciparum is also resistant to amodiaquine. (Beshir K et al., 2010). *In vitro* studies have shown that amodiaquine and chloroquine mode of action is by inhibiting hemozoin crystallization and glutathione-dependent destruction of ferriprotoporphyrin IX peptide in the *Plasmodium* parasite.

(Famin & Ginsburg 2002, German & Aweeka 2008). Currently amodiaquine is only recommended for treatment and not prophylaxis against malaria. This is owing to the rare but serious adverse effects such as hepatitis and agranulocytosis resulting from its long-term use. Amodiaquine was therefore re-introduced into the malaria chemotherapy and currently used as a partner drug for the artemisinin combination therapy with the short-lived Artesunate. The WHO recommended dosage is 10 mg/kg free base (13.5 mg/kg salt) (salt form is amodiaquine dihydrochloride) daily for 3 days usually in combination with other agents. (WHO 2016, Hoekenga et al., 1952). Combination therapy of sulfadoxine-pyrimethamine with amodiaquine has been reported to be efficacious regimen for intermittent preventive treatment to prevent malaria in children (IPTc) however the amodiaquine component is not always well tolerated. (Kerb R et al., 2009). Clinical studies in multicentered phase III trials have shown that the artesunate-amodiaquine combination therapy was more efficacious than amodiaquine monotherapy explored in African children, however cases of neutropenia were reported. (Adjuik M et al., 2002; Ndiaye JL et al., 2009).

Like chloroquine, amodiaquine is also administered orally and it is rapidly absorbed from the GI tract and undergoes rapid metabolism in the liver to N-desethyl-amodiaquine (DEAQ) that concentrates in the blood cells. Studies have reported that amodiaquine concentration in the blood is low however it is more potent than its metabolite, it is the DEAQ that's responsible for most of antimalarial activity. *P. falciparum in vitro* studies comparing both amodiaquine and its metabolite reported that amodiaquine enhanced DEAQ's efficacy and therefore showed a synergistic activity. Researchers are still investigating on other amodiaquine metabolites such as N-bis-desethyl-amodiaquine and 2-hydroxydesethylamodiaquine. (Winstanley et al., 1987; Mariga ST et al., 2004; Giao & de Vries 2001).

Even though clinical trials and studies have shown that artesunate-amodiaquine combination treatment as efficacious for uncomplicated *P. falciparum* malaria, some of the adverse effects associate with use of amodiaquine and that have been reported include: nausea, skin rash, itching, diarrhea,

agranulocytosis, hepatotoxicity, asymptomatic neutropenia and acute dystonic reaction to name a few (Winstanley PA et al., 1990). Some studies have suggested that toxicity with amodiaquine is directly dose-dependent besides involvement of host's immune response; while others have also reported that amodiaquine's reactive quinoneimine metabolite as the key toxic agent that triggers anti-amodiaquine IgG antibody-based immunological response. (Rhodes EG et al 1986, Rouveix B et al 1989).

## **Pyronaridine**

Recently, 4-aminoquinoline derivatives closely related to chloroquine and amodiaquine are shown to have potency even against CQ resistant strains of parasites (Kaschula CH et al., 2002; Raynes KJ et al., 1999). Pyronaridine, which is amodiaquine-like compound, was developed in China is now a marketed drug which has been through clinical trials for the combination therapy with artesunate (Ringwald P et al., 1996; Deshpande and Kuppast 2016). Pyronaridine (Malaridine), a benzonaphthyridine derivative, was first synthesized in China in 1970 where it has been used as antimalarial single agent for malaria treatment for nearly more than three decades ago, however it has very limited use in the rest of the world where malaria is endemic. Recently interest has been renewed in pyronaridine as a possible partner drug for ACT therapy and therefore it is considered an investigational drug for a fixed-dose combination with artesunate (3:1 ratio) for treatment of uncomplicated falciparum malaria and also blood stage vivax malaria. The investigational ACT is under the trademark known as Pyramax. (European Medicines Agency 2015; Ramharther M et al., 2008; Zheng XY et al., 1979, 1982].

Pyronaridine chemical formulae is as shown (4-[(7-chloro-2-methoxybenzo[b][1,5]naphthyridin-10-yl)amino]-2,6-bis[(pyrrolidin-1-yl)methyl] phenol). Pyronaridine is composed of a 9-aminoacridine (mepacrine) and amodiaquine-like structure (Chanc C et al., 1992; Chang YC et al., 2001). The salt form of the drug is known as pyronaridine tetraphosphate which constitutes about 56% parent or free base. It is yellow in color and odorless but bitter and it can be administered orally



as a tablet, intramuscularly in liquid form, and also via intravenous route. There are different versions of the drug in form of capsule formulation (100 mg and 50 mg free base), capsule fixed dose with Artesunate (3:1) coated tablet (Shao BR et al., 1990; Ramanathan S et al., 2005). The currently administered human dose in combination with artesunate (3:1) is 4 tablets of 180 mg pyronaridine tetraphosphate salt and 60 mg artesunate. This is 12 mg/kg of pyronaridine tetraphosphate and 4 mg/kg of artesunate each day for three days. Pyronaridine tetraphosphate salt is soluble in water (allows for effective oral absorption) while the free base is very sparingly soluble in water and it is more liposoluble than the salt. (Adegoke OA et al., 2006).

As a 4-aminoquinoline, pyronaridine acts by inhibiting heme crystallization. Studies have shown that pyronaridine interferes with the *Plasmodium* food vacuole of the late trophozoites and schizonts (Wu LJ et al., 1988; Kawai S et al., 1996). Other *in vitro* studies have also shown that it targets haematin formation by inhibiting beta-haematin formation with similar IC<sub>50</sub> of 0.125  $\mu$ M as chloroquine (Auparakkitanon S et al., 2006). Moreover, more studies have reported that pyronaridine inhibits glutathione-dependent haem degradation (Auparakkitanon S et al., 2006; Famin O et al., 1999). More investigations are on-going to fully elucidate on its mechanism of action. Studies using murine models have indicated that pyronaridine has potent activity against malaria asexual stages. Other studies have used the primate models to investigate on the schizontocidal activity of this 4-aminoquinoline and it was found efficacious against *P. inui* in rhesus monkeys. (Ye XY et al., 1990; Looareesuwan S et al., 1996).

Cross-resistance with chloroquine and other antimalarials studies have been done and it was determined that pyronaridine is more potent than chloroquine against *P. falciparum* and chloroquine-resistant isolates *in vitro* though this has been inconsistent (Childs GE et al., 1988; Ringwald P et al., 1996, Pradines B et al., 1999). Not clearly elucidated is the mechanism by which resistance to pyronaridine develops. However, a study shown that rodent *P. berghei* ANKA slowly developed resistance to pyronaridine given at low dose of 4 mg/kg (no detectable high resistance within 31-45

passages) however higher doses were more effective at selecting for resistance (Shao BR et al., 1986). In vivo resistance with combination therapy – researchers have derived pyronaridine-resistant *P. berghei* and *P. yoelii* strains by *in vivo* serial passage and shown delayed development of resistance in *P. berghei* versus pyronaridine alone (Peters W et al., 1992, 1999, 2000). These results are encouraging as they indicate that pyronaridine plus artesunate may retard the development of resistance. Resistance in clinical isolates from China where pyronaridine has been used for more than three decades now as both combination therapy and monotherapy, data has shown the potentiality of resistance developing but at a slower pace in spite of chloroquine resistance. Other studies have shown that the combination therapy of pyronaridine and other antimalarials potentially reduces pyronaridine resistance development (Yang H et al., 1998, Looareesuwan S et al., 1996).

## **ANTIMALARIALS PHARMACODYNAMICS / KINETICS**

Pharmacodynamics is the action of the antimalarial drugs against the *Plasmodium* parasites.

Pharmacokinetics is how the body handles or responds to the drug. Pharmacokinetics-

Pharmacodynamics (PK-PD) are some of the key determinants of a therapeutic response. The antimalarial drugs' speed of response in killing the parasites mirrors the rate at which the disease is controlled (White NJ 2013). Therefore, understanding the PK-PD of each antimalarial class will help with effective optimization of the drug dosage and quantitate therapeutic response. Currently, treatment of malaria relies on the combination therapies, even though effective dose, schedule and duration of the combination regimens is yet to be fully determined or elucidated. The PK-PD models have been used to help describe the parasitemia time-course in the malaria human patients and animal models before, during and post treatment with the antimalarial regimens given in combination or as monotherapy [Gordi T et al., 2005]. The ACTs have not been formulated based on suitable matching PK and PD profiles to improve efficacy and delay or prevent resistance. (WHO 2011).

Studies have been done to assess the pharmacokinetics and pharmacodynamics of the antimalarial agents individually looking at the drug's mechanism of action, clinical pharmacology – absorption, distribution, metabolism, elimination, and clinical pharmacodynamics. Studies have assessed PK properties of chloroquine, a 4-aminoquinoline, and found that it is well absorbed from the gastrointestinal tract and tissues, it is extensively distributed in the tissues such as liver, kidney, spleen, lung and melanin-containing tissues, it is nearly 60% bound to plasma proteins, it is metabolized to desethylchloroquine and chloroquine alone accounts for nearly more than 50% of urinary products while the metabolite accounts for only 25%. Chloroquine's elimination half-life has been reported to be in the range of 3 to 5 days while the terminal half-life is 30 to 60 days. [Brunton et al., 2010, Lexi Comp 2013]. For the clinical pharmacology, chloroquine is now resistant to *P. falciparum* in the malaria endemic countries but it still maintains activity against some strains and it has been found to be mostly effective against the asexual stages of *P. vivax*, *P. ovale* and *P. malariae*. (Brunton et al., 2010; Gao PT and de Vries PJ 2001; Moore BR et al., 2001).

Amodiaquine has also been found to be well absorbed from the gastrointestinal tract, the distribution volume range from 20-40 L/kg, it is metabolized to monodesethylamodiaquine though more metabolites are still being discovered, its elimination follows first order kinetics mean range of 5-8 hours for amodiaquine and 9-18 days for desethylamodiaquine. Amodiaquine is currently given in combination with artesunate as one of the recommended ACTs by the WHO since it is active against chloroquine-resistant strains. (Brunton et al., 2010; Gao PT and de Vries PJ 2001).

Artesunate, an endoperoxide, which is currently the first-line recommended partner drug for ACTs, has a shorter half-life compared to the 4-aminoquinolines and it is also rapidly metabolized to its active metabolite known as dihydroartemisinin (DHA) that concentrates in the infected erythrocytes, artesunate absorption is not affected by food and it is about 43-82% bound to plasma proteins. Elimination studies have also suggested that less than 1% of the drug is recovered as parent drug in urine, feces and bile. Artesunate oral bioavailability is about 15% compared to intravenous route and the half-life is nearly 2.7 minutes post intravenous administration while reported value for its metabolite DHA is about 40 minutes after oral or intravenous routes. (Brunton et al., 2010; Gao PT and de Vries PJ 2001, Morris CA et al., 2011).

Pyronaridine, which is currently considered the most interesting 4-aminoquinoline for the ACTs, needs further extensive pharmacological studies on its PK / PD properties. It might be considered as first-line for treatment of uncomplicated malaria in the endemic regions with chloroquine-resistance. Clinical studies have assessed pyronaridine's pharmacokinetics and found that intramuscular dose is absorbed with T<sub>max</sub> 0.66 hours and C<sub>max</sub> 525 ug/L while T<sub>max</sub> post oral administration of 600 mg salt was 5 to 14 hours (Fu S 1991; Chang C et al., 1992), and it has a terminal half-life of about 64 hours. Other studies such as the Sang & Pradeep 2009 assessed the absorption, distribution, excretion and PK properties of pyronaridine tetraphosphate using rat models. This group found that following oral administration of single dose 10 mg/kg salt the drug was readily absorbed from the small intestine within 1 hour and widely distributed in most of the

tissues with drug peak in blood was reached at 8 hours post administration. This group also found that the excretion of the drug was through urine at peak excretion post 24 hours of administration, the C<sub>max</sub>, AUC and T<sub>max</sub> values were similar to those observed in the clinical phase II trials of artesunate-pyronaridine conducted in Uganda. The plasma parameters post dosing with the 10 mg/kg pyronaridine salt were C<sub>max</sub> 189-207 ng/ml, T<sub>max</sub> 7-8 hours, half-life 148-157 hours (6-6.5 days) and AUC<sub>240hr</sub> was about 14 ug.hg/ml while at infection was about 20 ug.hg/ml. The blood parameters were nearly similar to the plasma parameters. [Park SH 2010; Davis CB et al., 2008).

Pharmacokinetics assesses the time course of the antimalarial drug and its effects in the body, besides it also allows for the quantification of absorption, distribution, metabolism and excretion post taking the drug. These are some of the processes that help determine the drug concentration in the body, for instance, in whole blood and in plasma. An effective dosage regimen is determined by the concentration of the antimalarial drug in the body. Drug concentrations are mostly measured in whole blood, plasma and serum besides other body fluids. The measured drug concentrations are referred to as drug levels, the total drug concentration (both bound and unbound drug in equilibrium). The concentration of the drug in the plasma is not usually the same as the concentration in different organs and tissues. There are different pharmacokinetic models as described herein. The unimodal response is whereby the drug achieves a homogenous distribution throughout the body and then it equilibrates in all the tissues. This concentration-time profile would show a log linear correlation. The bimodal and multimodal response is when the drug does not achieve distribution in the different organs and tissues in the body instantly, hence the concentration-time profile would show a curve with initial rapid decline in concentration and then it flattens out – equilibrates to other different tissues. The rapid decline in concentration is due to distribution to other tissues and organs as well as elimination from the body. (Winter 2003; White & Garrison 1994).

The pharmacokinetics parameters in human models have shown that the 4-aminoquinolines – chloroquine, pyronaridine and amodiaquine have an apparent clearance range from 0.1- 0.78, 0.72

and 5.52-17.1 L/h/kg respectively. Both amodiaquine and chloroquine have been shown to have oral bioavailability of nearly 90% while artesunate 15-32 %. (Table ii). Chloroquine has been shown to have a large volume of distribution (100-1000 L/kg) compared to artesunate which has zero volume of distribution. (Table ii).

**Table (ii): Artesunate & 4-aminoquinolines PK parameters in humans**

<b>Drug (base)</b>	<b>Oral bioavailability (%)</b>	<b>Protein binding (%)</b>	<b>Volume of distribution Vd (L/kg)</b>	<b>Apparent Clearance (CL) L/h/kg</b>	<b>Half-life hours</b>	<b>Main metabolite</b>	<b>Reference</b>
<b>Artesunate</b> 120mg i.v	15		0	2.33	2.7 min	DHA	Batty et al., 1998
<b>Amodiaquine</b> (200-600 mg oral)	80-90	>90	17-38	5.52-17.1	2-10	Monodesethyl	Winstanley PA et al., 1987
<b>Chloroquine</b> (300 mg oral)	80-90	50-64	100-1000	0.1-0.78	40-55	Mono/bis desethyl	De Vries et al., 1994; Ette El et al., 1989
<b>Pyronaridine</b> (600mg IM)	19-32	>65	71	0.72	64	None	Chang C et al., 1992; Fu & Xiao 1991

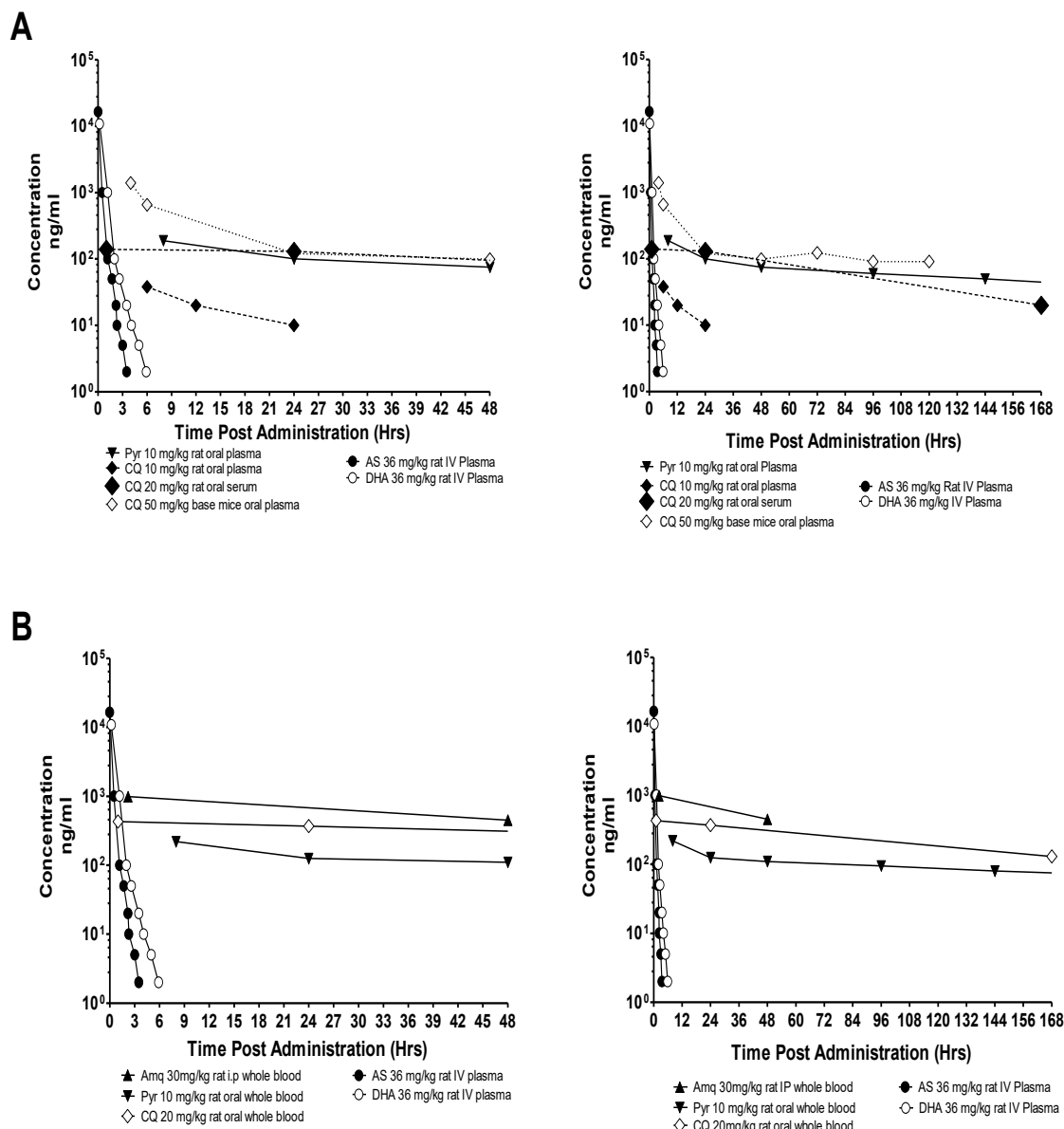
Key Reference article: Giao PT and de Vries PJ. 2001. Pharmacokinetic Interactions of Antimalarial Agents. *Clin Pharmacokinet* 40 (5): 343-373.

Besides looking at the pharmacokinetics parameters in humans (Table ii), other studies have also assessed the plasma and whole blood concentration-time profiles of artesunate and the 4-aminoquinolines using rodent models (Figure ii). Studies assessing the PK parameters for pyronaridine salt 10 mg/kg administered orally to rat models have reported nearly the same parameters in whole blood and plasma concentration-time profiles. These findings were nearly similar to findings observed in clinical phase II trials in humans. (Park SH 2010; Davis CB et al., 2008). Studies that assessed artesunate and its metabolite dihydroartemisinin (DHA) administered in rats at 36 mg/kg intravenously showed that for both artesunate / dihydroartemisinin (DHA) the concentration declined faster and near complete elimination was achieved within 3 to 6 hours post administration however in clinical studies have reported much shorter half-life (nearly 3 minutes) compared to animal studies. The 4-aminoquinolines compared to artesunate, maintain a high

concentration level ranging between 100-1000 ng/ml in both the whole blood and plasma which allows for their long-acting activity post administration. The 4-aminoquinolines are widely distributed throughout the body compared to artesunate/DHA. For instance, chloroquine and amodiaquine shows a bimodal response in the concentration-time profiles with a sharp drop followed by slow decline. (Figure ii).

For the quinolones there is confusion in dosing on amount of salt and base given which can lead to under or overdosing adults and children. Table (iii) shows the normal adult total daily dose and total dose over three days for artesunate and the quinolones. These daily doses can be converted to a mg/kg for pediatric dosing and conversion for animal or rodent doses which are allometrically equivalent to human dosing. In general when converting a human mg/kg dose to mouse it is necessary to multiply by a factor of 12 such that a 10 mg/kg human dose with chloroquine base is equal to 120 mg/kg in the mouse.

Figure (ii): Plasma (A) and Whole Blood (B) Concentration-Time Profiles for artesunate, pyronaridine, chloroquine and amodiaquine in murine models.



Reference: Netty H et al., 2017; Adan-abdi Y et al., 1985; Moore BR et al., 2001.



**Table (iii). Human to mouse base and salt drug conversions**

drug	Daily human dose mg Base	Daily human dose mg Salt	total treatment dose mg Base	total treatment dose mg Salt	Daily human mg/kg base	Daily human mg/kg salt	Allometric mouse mg/kg base	Allometric mouse mg/kg salt
artesunate	240	240	720	720	4	4	48	48
pyronaridine tetraphosphate	400	720	1200	2160	6.7	12	80	144
chloroquine diphosphate	600*	1000*	1500	2500	10	16.6	120	200
amodiaquine dihydrochloride	600	790	1800	2370	10	13.2	120	160

\*chloroquine-900 mg base day 1, 300 mg base day 2 and 3

## **CHALLENGE, RATIONALE, HYPOTHESIS & AIMS**

### **Challenge**

Humans with a 4% parasitemia have about one trillion parasites (4 million erythrocytes per microL of blood or a 4 trillion per liter times 6 liters times 0.04 parasitemia = one trillion infected erythrocytes per adult). Human malaria chemotherapy can be measured by a parasite reduction per time interval such as the 48 hour single *P. falciparum* life cycle. The artemisinins are postulated to provide a 4 log killing rate each 48-hr parasite cycle, quinolines with a 3 log killing rate, antifolates with 2 logs and antibacterials with a single next cycle (delayed generational) 1 log kill rate (Nicholas White 2004). In contrast to this parasite reduction measurement in humans, drug screening *in vitro* with *P. falciparum* starts with more than a billion times less parasites (about 100 thousand) to compare percent inhibition with untreated drug control which increases about 10-100 fold at 72 hrs. Parasites that slowly increase a log with untreated parasites increasing 2 logs have 90% inhibition. Most mouse models of drug activity commonly utilize the Peters suppression test which delivers a low parasite inoculum of parasites followed by high daily doses of drugs commencing hours after infection (W Peters 1974). The murine malaria suppression tests inoculates 500,000 to 1 million parasites followed immediately by high drug doses. Parasite number at 4 days on drug is compared to no drug controls which multiply by a log each day or by about 4 logs in 4 days. If drug treatment results in a slower growth at only 2 logs in 4 days, this is expressed as 99% inhibition even if the parasites have multiplied.

The majority of previous laboratory based malaria drug studies have relied on drug inhibition of low numbers of parasites numerically compared to untreated controls. In contrast, human malaria drug studies measure the decline or killing in high numbers of parasites numbering near a trillion per person. Therefore, for this thesis research I have adapted a murine malaria model utilizing a luciferase reporter rather than blood film counting to measure the decline in parasitemia after single drug dosing as a measure of killing compared to starting parasite number rather than inhibition compared to untreated control growth.

## Rationale:

The previous malaria drug screening efforts mostly focused on the inhibition assays done *in vitro* and *in vivo*, low parasite inoculum, high drug dose and comparison to no-drug control. Now my work mainly focuses on the newer pharmacodynamics killing assay with a high parasitemia burden and clinically relevant drug dosing to measure speed of drug response in killing parasites, which is important to quantitate therapeutic responses with different antimalarials.

## Hypothesis

Single dose or combination therapy pharmacodynamics of *Plasmodium* drug killing in a high parasitemia *in vivo* model is different from parasite inhibition *in vitro*.

## Research Aims

**Aim 1:** To compare single oral dose dynamic killing with Artesunate and 4-Aminoquinolines (Pyronaridine, Chloroquine, Amodiaquine).

**Aim 2:** To measure 8 hour, 24 hour and 48 hour killing by the long half-life 4-aminoquinolines in an infected blood transfer experiment where long exposure quinolone drugs are washed away.

**Aim 3:** To investigate combination killing profiles with single dose 4-aminoquinolines (chloroquine, pyronaridine and amodiaquine) and Artesunate.

The pharmacodynamic parasitocidal metrics employed are parasite log reduction, undetectable parasite killing duration, speed & duration to recrudescence (initial parasitemia) and 30-day cure. The outcome of this work is a novel ranking of malaria drugs by parasitocidal properties rather than inhibition properties. This thesis work will also validate the model as a potential higher throughput model for drug killing, which is not readily measurably achievable with *P. falciparum* in culture (RD Young 1993). We argue that *P. falciparum* *in vitro* inhibition assays are not killing studies. We therefore hypothesize that kinetics and synergy of killing will be different from inhibition studies as recently shown for chloroquine alone (MF Paguio 2011).

## CHAPTER 2 – METHODOLOGY

### **Antimalarial Drug Preparation**

The antimalarial drugs artesunate and the 4- aminoquinolines-pyronaridine, chloroquine and amodiaquine were obtained from Sigma Aldrich. The quinoline salts used were pyronaridine tetraphosphate (MW = 910 and 56% free base), chloroquine diphosphate (MW = 515 and 62% free base) and amodiaquine dihydrochloride (MW = 464 and 76% free base). Artesunate is 100% parent drug. For all the experiments, the quinolines were dosed in salt form. The conversion of the base / salt weight percentage is shown on table (iv) indicating the actual measurement of free base in the salt dosage employed and showing the mouse and human dose equivalents. Artesunate was dissolved in 5% sodium bicarbonate, pyronaridine tetraphosphate, chloroquine diphosphate and amodiaquine dihydrochloride were each dissolved in deionized water (dH<sub>2</sub>O). Fresh drug solution was used for each experiment. Each drug solution was dosed at volume of 200  $\mu$ L per mouse.

### **Parasiticial *in vivo* Murine Malaria Model**

All the experiments were done under the approved protocol MO15H319 by the Johns Hopkins University's Animal Care and Use Committee. The rodent model used for all the experiments were Balb/cj female mice from the Jackson Laboratories aged at least 6 weeks old and weighed approximately 20 grams each. A replicate of three mice were used for each drug dose regimen tested.

### **Infection with *Plasmodium berghei* ANKA GFP-Luciferase strain**

*P. berghei* ANKA, 676m1cl1, Green fluorescence protein-luciferase (PbANKA GFP-Luc) obtained from ATCC (catalog # MRA-868) constitutively expresses the luciferase at all stages in the life cycle. The PbANKA GFP-Luc clone enables 4-8 hour precise measurements of parasite dynamics over a 3 log range in a IVIS Spectrum Imager using the plate reader function. For each experiment Balb/cj female mice were infected via intrapetronal (i.p) with a 10,000 infected erythrocytes from donor mouse in the first or second passage. The isolate obtained from the BEI ATCC was passaged through mosquitoes, infecting donor mice via liver then blood stage, which

were then exsanguinated, aliquoting infected blood for low (less than three) passage number in all experiments. Parasitemia in the experimental mice was monitored using Giemsa-stained thin blood film counting and luciferase measurements until approximately 3 to 4 % parasitemia was reached before initiating drug dosing - translating to millions of parasites per microliter as will be described in the luciferase assay protocol below.

### **Parasite Monitoring**

Initial single (or multi) dose drug treatment with artesunate and the 4-aminoquinolines begins on day four (0-hr with high density parasitemia-millions parasites per microliter) post infection. Blood was withdrawn at 0-hr, 3-hr, 6-hr, 12-hr, 24-hr time-points within the first day post dose and followed up every day for 30 days post dose or until initial starting parasitemia was reached upon which mice were euthanized following the standardized protocol at the animal facility situated at the Johns Hopkins School of Public Health.

### **Luciferase Reporter Assay**

Our murine malaria model utilizes a luciferase reporter assay rather than blood film counting to measure the decline in parasitemia after drug dosing. Luciferase reporter assay is a cell-based assay that analyzes the intracellular infection mechanisms of the parasite before and after drug treatment. The *Plasmodium* expressed firefly luciferase catalyzes luciferin oxidation using ATP-Mg<sup>2+</sup> as co-substrate. After the enzyme and substrates combine, a flash of light is generated and decays rapidly. The assay yields linear results over at least 4 orders magnitude. The assay is also comparable to the Giemsa staining in that it also measures the total number of parasites present at different end-points and it enables more time points than blood film counting.

### **Blood Sample Collection**

For the blood sample collection, 5 µL of mouse tail blood is plated into 45 µL lysis buffer in a white round bottom 96 well plate stored at -80C (*Lysis buffer reagents: 20mM Tris (pH 7.5), 5mM EDTA, 0.008% (wt/vol) saponin, and 0.08% (vol/vol) Triton-X-100*) (Franke-Fayard B 2008). Then 5 µL of diluted lysed blood (0.5 µL of whole blood equivalent) was placed in black opaque 96-well plate

and 95  $\mu\text{L}$  of the luciferase buffer was added (1:20 ratio of blood sample in lysis buffer to the FLAR reagents) then the luciferase activity was measured in the IVIS Spectrum In Vivo Imaging system then analyzed using the Living Image v.4.4 software to get the total flux (photon per seconds). Our model begins to dose drugs four days after infection with high parasitemia translating to billions of parasites per mouse (approximately 10% parasitemia) and which will measure malaria metrics: drug speed of parasite killing, length of undetected parasitemia, duration & speed of recrudescence (return to initial parasitemia) and 30-day cure.

### **Blood Transfer Experiment**

Balb/cj female mice were infected with *PbANKA*-GFP-Luc were dosed with specific single and multi-dose regimen of 4-aminoquinolines and artesunate respectively. Blood transfer time-points done were 0-hr, 8-hr, and 24-hr for all the drugs tested. Additional blood transfers done specifically for pyronaridine drug salt 50 mg/kg (30 mg/kg free base) were: 4-hr, 48-hr and 72-hr. At each transfer time point, 100  $\mu\text{L}$  (tail blood) of the infected blood was withdrawn from each treated donor mouse and diluted in 1000  $\mu\text{L}$  sterile PBS solution. The blood solution was then washed thrice with 1000  $\mu\text{L}$  sterile PBS by centrifugation (r.c.f. 3000 xg). After the third wash the infected erythrocytes were suspended in a final volume of 210  $\mu\text{L}$  and 5  $\mu\text{L}$  was placed in 45  $\mu\text{L}$  lysis buffer for quantification of transferred cells using luciferase assay. Two-hundred microliter was injected via i.p into uninfected mice in order to effectively lower drug concentration below minimal inhibition to determine cell viability. The following malaria metrics were followed: parasitemia growth curves, time to parasite number of 10,000 and 100,000 parasites per microliter post each transfer, duration of undetected parasitemia (below limit of quantification 1000 parasites /  $\mu\text{L}$ ) and time to starting parasitemia to compute number of killed parasites. Dilution curve of untreated infected mice at time measure served as the control.

## Data Analysis

All the data analyses and representations were performed with Graph Pad Prism 5 Software. Data are represented as mean  $\pm$  SEM. The luciferase raw data log 10 total flux (photons/sec) was transformed to parasites per microliter using equation  $Y = (10^{((\log(y)-0.55)/0.05)})^2$  (Walker & Sullivan, 2017).

All data were normalized to geo-mean starting parasitemia of 5 million parasites per microliter.

Parasite log reduction (Pi – Pt) was achieved by getting the difference between Log10 of initial parasitemia (6.7) and parasitemia at time-point (t) – 6-hr, 12-hr, 24-hr and 48-hr. The statistical methods employed include the following: Two-Way ANOVA and / or t-test (non-parametric) were used for comparing two groups. For groups more than two, One-way Analysis of Variance (ANOVA) with Turkey Multiple comparison post-hoc test were used for comparison of groups as appropriate with alpha significance level  $p < 0.05$ .

**Table (iv): Mouse doses and human equivalents of artesunate and 4-aminoquinolines**

Antimalarial Drug	Mouse Dose reference body weight 20 gm (Free Base)		Human Dose Equivalent reference body weight 60 kg, multiply mouse dose by 0.081 factor (Free Base)	
Artesunate	10 mg/kg		0.8 mg/kg	
	50 mg/kg		4 mg/kg	
	100 mg/kg		8 mg/kg	
	250 mg/kg		20 mg/kg	
Chloroquine diphosphate	10 mg/kg	(6)	0.8 mg/kg	(0.5)
	50 mg/kg	(30)	4 mg/kg	(2.5)
	100 mg/kg	(60)	8 mg/kg	(5)
	250 mg/kg	(150)	20 mg/kg	(12)
Amodiaquine dihydrochloride	10 mg/kg	(8)	0.8 mg/kg	(0.6)
	50 mg/kg	(40)	4 mg/kg	(3)
	100 mg/kg	(75)	8 mg/kg	(6)
	250 mg/kg	(190)	20 mg/kg	(15)
Pyronaridine tetraphosphate	1 mg/kg	(0.6)	0.08 mg/kg	(0.05)
	2.5 mg/kg	(1.5)	0.2 mg/kg	(0.11)
	5 mg/kg	(3)	0.41 mg/kg	(0.23)
	7 mg/kg	(4)	0.57 mg/kg	(0.32)
	10 mg/kg	(6)	0.8 mg/kg	(0.5)
	50 mg/kg	(30)	4 mg/kg	(2.2)
	100 mg/kg	(60)	8 mg/kg	(4.5)
	150 mg/kg	(90)	12 mg/kg	(6.8)
	250 mg/kg	(150)	20 mg/kg	(11)

Reference: [USFDA 2005]. Human equivalent dose calculation factor obtained from FDA draft guidelines. FDA: Food and Drug Administration, HED: Human equivalent dose. Nair AB, Jacob S. A simple practice guide for dose conversion between animals and human. J Basic Clin Pharma 2016;7:27-31. The value in brackets denotes free base dose of that specific drug.

## CHAPTER 3 – RESULTS

**Artesunate exhibits dose-dependent killing in a single oral dose regimen.** To investigate the single dose dynamic killing with artesunate, we tested dose regimen escalation at 10 mg/kg, 50 mg/kg, 100 mg/kg and 250 mg/kg in a *PbANK4*-GFP-Luc infection. The six and 12 hour log reduction was similar except 10 mg/kg regardless of the dose at about 1 log reduction in this short time period. In contrast the 24 and 48-hr measurement show a clear progressive larger log reduction with increasing artesunate dose. The human dose of 4 mg/kg is equivalent to 50 mg/kg in mice and just showed a single log reduction at the full 24-hr cycle in *P. berghei*. The duration to return in initial parasitemia was also progressive with increasing doses and no cure. (Figure 1a, Table 1a).

**Pyronaridine exhibits dose-independent killing in a single oral dose regimens.** Pyronaridine, a 4-aminoquinoline, was also tested at 10 mg/kg, 50 mg/kg, 100 mg/kg and 250 mg/kg given as pyronaridine tetraphosphate salt (Figure 1b, 2a). Pyronaridine exhibits dose-independent killing and completely cures at all doses (Figure 1a). While the doses for pyronaridine are based on the total salt, the active molecule, the free base is 56% of the salt, which almost doubles the potency (Table (ii) in methods). Parasite log reduction at 6 hours was similar to artesunate at 1 log reduction but by 12 hours post dosing was measured near 2 log reduction. At 24 hours post dosing, there was a large 3-4 log reduction increasing to nearly 5 - 6 log drop at 48 hours post dosing. (Table 2b). All the mice (n=3) in each of these dose groups were completely cured at 30 days.

**Chloroquine and amodiaquine exhibits initial dose-independent killing in a single oral dose regimen.** Chloroquine and amodiaquine, also 4-aminoquinolines, were tested with a dose regimen administered at 10 mg/kg, 50 mg/kg, 100 mg/kg and 250 mg/kg based on salt weight (chloroquine base is 62% of salt; amodiaquine base is 76% of salt). Chloroquine and amodiaquine show a plateau phase after an initial 6 hour drop from 6 to 24 hours post dosing with an approximate single log reduction the first 24 hours no matter the dose from 10 to 250 mg/kg (Figure 1c-d). Mice dosed with 50-250 mg/kg showed a further dose independent ~3 log parasite reduction falling near the limit of quantification (1000 parasites/ $\mu$ l) at day 3 after initial dose before



reversing. (Table 1c-d). Measure of duration to return to initial parasitemia was dose-dependent taking nearly 3- 9 days for mice to return to initial parastemia with increasing doses, a reflection of longer drug exposure time. Two of three mice achieved complete cure with amodiaquine 250 mg/kg.

**Single oral low dose pyronaridine at 10 mg/kg or below is more potent than artesunate administered in a total dose of 150 mg/kg and 600 mg/kg sustained over 24 hours in a 3-dose regimen.** We were interested in comparing the single dose killing dynamics of low dose pyronaridine 1-10 mg/kg to artesunate given in three dose regimen of 50 mg/kg every 8 hr totaling 150 mg/kg and also 200 mg/kg every 8 hr totaling 600 mg/kg given at time-points 0, 8 and 16 hours post initial dose, respectively (Figure 2a,b). Artesunate has a short 6-8 hour exposure in the mouse, so testing a multiple dose regimen over 24 hours creates a sustained drug level comparable to the quinolines. Pyronaridine at single dose of 1 mg/kg showed no reduction. However, 5 mg/kg and 7 mg/kg had nearly 1 log kill at 24 hours and nearly 2 log kill at 48 hours post initial dosing. Indeed the 5 mg/kg pyronaridine dose was similar to the 250 mg/kg artesunate single dose in Figure 1a. Two of three mice with single dose pyronaridine at 7 mg/kg achieved complete cure. However, single dose pyronaridine at 10 mg/kg was most potent with nearly 3 log kill at 24 hours and 5 log kill at 48 hours, almost twice the log drop seen with 5 mg/kg and 7 mg/kg, post initial dosing. (Table 2a). All the mice dosed with 10 mg/kg pyronaridine were cured. Mice that received 3-dose regimen of artesunate at 200 mg/kg a dose (600 mg/kg total) (Figure 2b) showed a rapid reduction in parasites with a log reduction of 3 and 5 similar to as single dose 10 mg/kg pyronaridine at 24 hours and 48 hours post initial dose, respectively. (Table 2b). The duration to return in initial parasitemia with low dose pyronaridine was dose-dependent from 3 to 11 days. The 50 mg/kg each dose (150 mg/kg total dose) also showed a large log reduction in parasite numbers with 2 logs at 24 and 3.5 logs at 48 hours more comparable to the pyronaridine numbers.

Following single dose dynamic killing profiles we were interested in investigating on the measure of 8-h and 24 hours killing by the long-half life 4-aminoquinolines and short half-life artesunate in an infected blood transfer dilution in order to effectively lower the drug concentration

below minimal inhibition. In this experiment, (Figure 3a-c) pyronaridine 50 mg/kg shows a linear correlation between the parasite log drop and duration it takes to reach 100,000 viable parasites post transfer at hours 4, 8, 24, 48 and 72 respectively. Both low dose (50 mg/kg) and high dose (150 mg/kg) pyronaridine treated blood took nearly the same number of days to reach viable parasites post 8-and 24 hour transfers and nearly same mean parasite log reductions. Only two mice came up positive during 48-hours transfer and one mouse during 72-hours transfer. (Figure 3a) Artesunate given in multi-dose regimen took nearly five days post 24-h transfer to reach viable parasites. Multi-high dose artesunate resulted in increased parasite log reduction almost below limit of quantification at 24-hrs and it took nearly 5 days to reach viable parasites post transfer into uninfected mice. (Table 3). High dose AS at 24-hour transfer took nearly the same amount of days to reach viable parasites as single dose pyronaridine 50 mg/kg at 48-hour transfer. (Figure 3b). Chloroquine and amodiaquine at both 50 mg/kg single oral dose had nearly the same parasite log reduction at both 8 and 24 hours post dosing in the treated donor mice and the duration to reaching viable parasites post dilution transfer was not that different, took about 1 and 2 days post 8 and 24 hours transfers respectively. (Figure 3c, Table 3).

**Effect of single dose dynamic killing in combination therapy with artesunate and 4-aminoquinolines.** We were interested in investigating on the effect of single dose combination therapy with artesunate and pyronaridine, chloroquine and amodiaquine administered at 50 mg/kg each in the combination regimen (Figure 4a,b,c). Dose of 50 mg/kg is the mouse equivalent to 4 mg/kg artesunate administered in humans.

**Artesunate-pyronaridine combination (Figure 4a),** Artesunate 50 mg/kg alone had parasite log reduction of 1 log kill over 24 hours post dosing. At 24 hours and 48 hours post dosing, pyronaridine's parasite log reduction was 4 times and 6 times that of artesunate 50 mg/kg alone. However, in combination therapy with artesunate there was slight weak antagonism effect with a

smaller log kill at all time points past 12 hours. (Table 4a). Mice dosed with pyronaridine 50 mg/kg alone and in combination with artesunate 50 mg/kg were all cured.

**Artesunate-chloroquine or amodiaquine combination (Figure 4b),** In contrast to pyronaridine, chloroquine or amodiaquine and artesunate were additive in the interaction when dosed together. There was no plateau phase in combination killing observed between 6 and 24 hours compared to single dose chloroquine 50 mg/kg alone. Mice dosed with combination regimen at 50 mg/kg showed decrease in parasites slightly below the limit of quantification on day 3 then return to initial parasitemia nearly 6 days post initial dose (Table 4b,c). The artesunate-chloroquine combination therapy was not curative however there was a slight prolongation of duration to return to initial parasitemia.

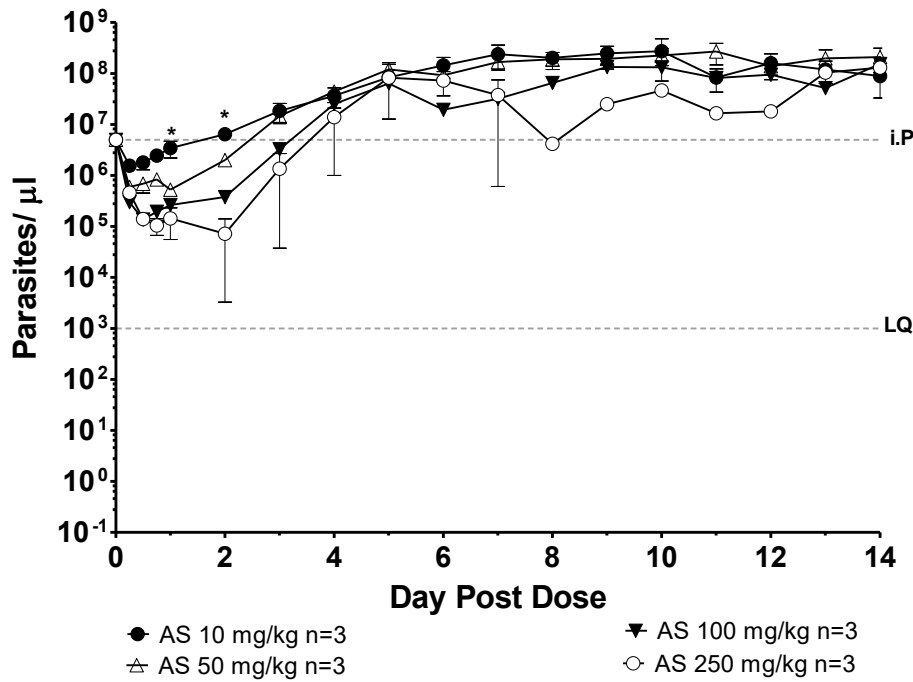
Comparing the combination drugs together in a single graph shows for the first 24 hours all have a similar 2 log reduction which slowed the next 24 hours with amodiaquine, was approximately 2 again for chloroquine and continued to a 5 log reduction at 48 hours for the pyronaridine combination. (Figure 4d, Table 4d).

**Effect of increasing artesunate dose in a single dose combination therapy with long-acting 4-aminoquinolines.** We were then interested in further comparisons of the effect of increasing the artesunate dose to 250 mg/kg while keeping the quinolines at 50 mg/kg to compare to 50 mg/kg for both combinations. Artesunate 250 mg/kg via i.p alone had parasite log reduction of 2 log kill and nearly 4 log kill at 24 hours and 48 hours post dosing respectively. However, single oral dose pyronaridine 50 mg/kg had almost twice artesunate's log kill at 24 hours and 6 log kill at 48 hours post dosing. Interestingly, in combination regimen with artesunate the parasite log reduction at time-points 6, 24 and 48 hours post dosing was similar to that of artesunate 250 mg/kg alone (figure 5a). This clearly shows presence of slightly weak antagonism effect. Mice dosed with combination therapy and single pyronaridine 50 mg/kg alone were all cured. The combination of chloroquine or amodiaquine and high dose artesunate had a similar log reduction with a lower nadir and delay in return to parasitemia with the combination therapy at 7-9 days

compared to chloroquine, amodiaquine or artesunate alone at nearly about 5 days (table 5b and c). Both chloroquine and amodiaquine displayed an additive effect of the drug combination on the rate of kill and delay in return to initial parasitemia. Interestingly, single oral dose pyronaridine 50 mg/kg given alone (Figure 5a) was still most potent than the other combination regimens (Figure 5d).

**Figure 1(a): Single Oral Dose Killing Profile of Artesunate in a *P.berghei*ANKA-GFP-**

**Luciferase infection.** Single dose artesunate dose regimen of 10 mg/kg, 50 mg/kg, 100 mg/kg and 250 mg/kg was administered orally to Balb/cj female mice (n=3) at time 0-hr which corresponds to ~ 10% parasitemia confirmed by Giemsa blood film counting. Data is represented as mean  $\pm$  SEM. One-way analysis of variance (ANOVA) p-value summary \*p-value<0.05.

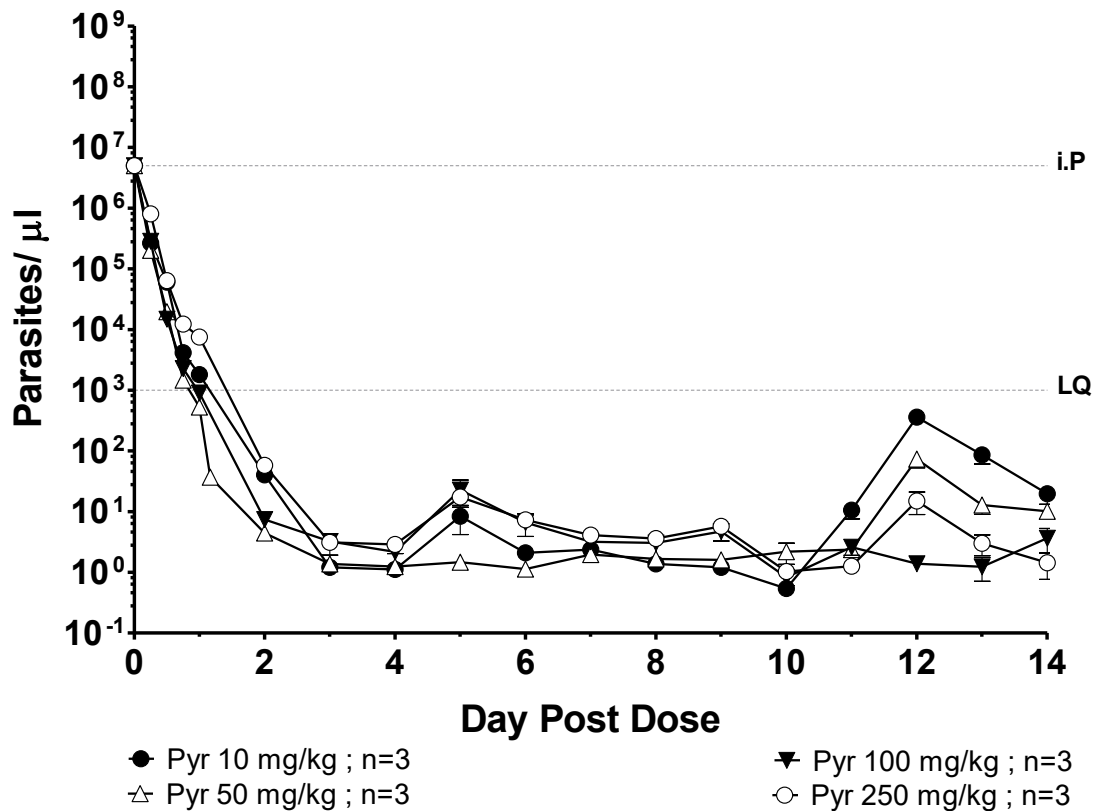


**Table 1(a): Measure of Parasite Log Reduction and Duration to Return to initial parasitemia (\*p-value<0.05)**

Dose (mg/kg)	Frequency	6-hr log-reduction	12-hr log-reduction	24-hr log-reduction	48-hr log-reduction	Days to return in initial parasitemia	Mice Cured
10	1	0.5	0.5	0.2	-0.1	1.5	0
50	1	0.9	0.9	1.0	0.4	2.5	0
100	1	1.2	1.2	1.3	1.1	3.2	0
250	1	1.0	1.0	1.5	1.8	3.5	0

**Figure 1(b): Single Oral Dose Killing Profile of Pyronaridine in a *P.berghei*ANKA-GFP-**

**Luciferase infection.** Single dose pyronaridine tetraphosphate salt dose regimen of 10 mg/kg, 50 mg/kg, 100 mg/kg and 250 mg/kg was administered orally to Balb/cj female mice (n=3) at time 0-hr which corresponds to ~ 10% parasitemia confirmed by Giemsa blood film counting. Data is represented as mean  $\pm$  SEM. One-way analysis of variance (ANOVA) p-value >0.05

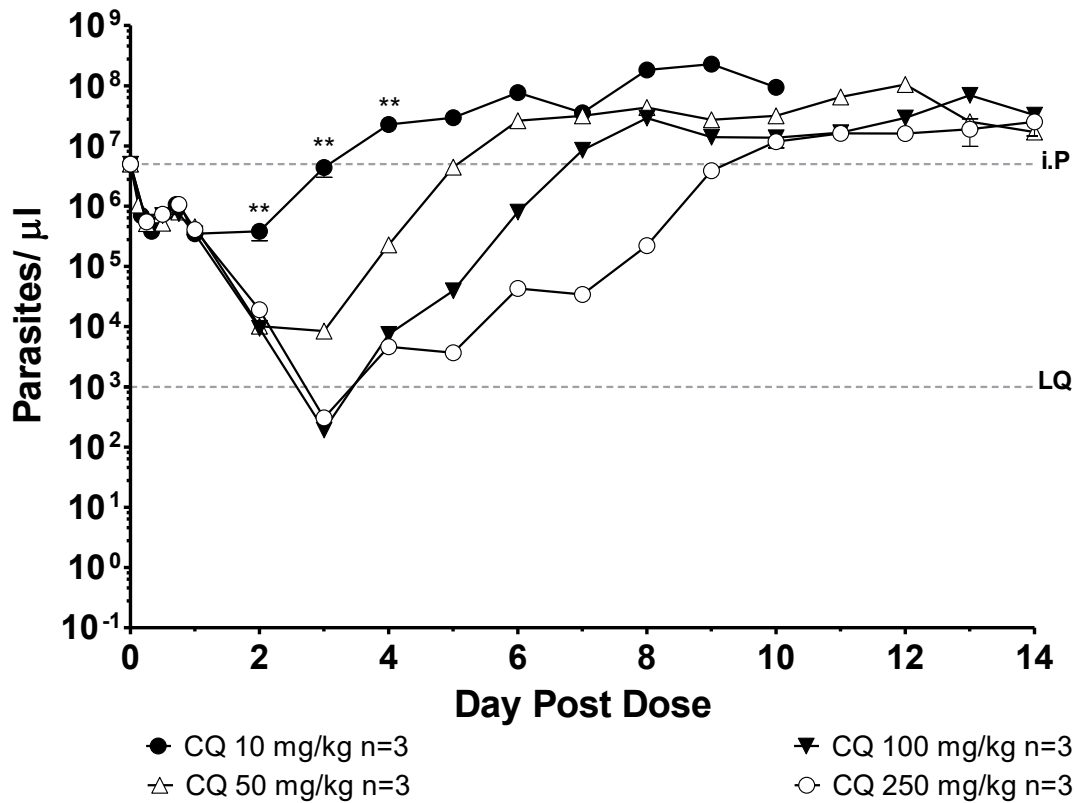


**Table 1 (b): Measure of Parasite Log Reduction and Duration to Return to initial parasitemia. ANOVA p-value>0.05 at each time point.**

Dose (mg/kg)	Frequency	6-hr log-reduction	12-hr log-reduction	24-hr log-reduction	48-hr log-reduction	Days to return in initial parasitemia	Mice Cured
10	1	1.3	1.9	3.4	5.1	CURE	3/3
50	1	1.4	2.4	4.0	6.1	CURE	3/3
100	1	1.2	2.5	3.7	5.8	CURE	3/3
250	1	0.8	1.9	2.8	4.9	CURE	3/3

**Figure 1(c): Single Oral Dose Killing Profile of Chloroquine in a *P.berghei*ANKA-GFP-**

**Luciferase infection.** Single dose chloroquine phosphate salt dose regimen of 10 mg/kg, 50 mg/kg, 100 mg/kg and 250 mg/kg was administered orally to Balb/cj female mice (n=3) at time 0-hr which corresponds to ~ 10% parasitemia confirmed by Giemsa blood film counting. Data is represented as mean  $\pm$  SEM. One-way analysis of variance (ANOVA) \*\*p-value <0.001

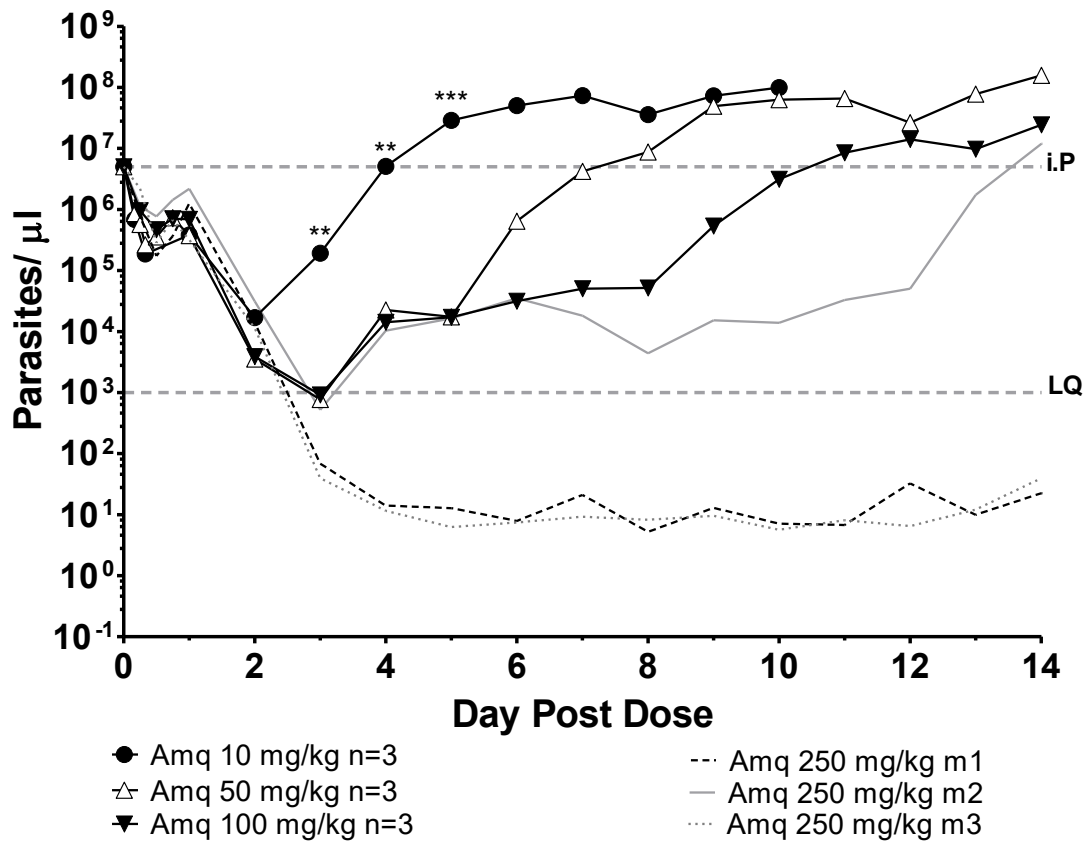


**Table 1 (c): Measure of Parasite Log Reduction and Duration to Return to initial parasitemia. ANOVA \*\*p-value<0.001**

Dose (mg/kg)	Frequency	6-hr log-reduction	12-hr log-reduction	24-hr log-reduction	48-hr log-reduction	Days to return in initial parasitemia	Mice Cured
10	1			1.2	1.1 **	3	0
50	1	0.6	0.9	0.9	2.5	4.8	0
100	1	1.0	0.9	1.2	2.7	6.8	0
250	1	0.7	0.8	0.9	2.5	9	0

**Figure 1(d): Single Oral Dose Killing Profile of Amodiaquine in a *P.berghei*ANKA-GFP-**

**Luciferase infection.** Single dose amodiaquine dihydrochloride salt dose regimen of 10 mg/kg, 50 mg/kg, 100 mg/kg and 250 mg/kg was administered orally to Balb/cj female mice (n=3) at time 0-hr which corresponds to ~ 10% parasitemia confirmed by Giemsa blood film counting. Data is represented as mean  $\pm$  SEM. ANOVA \*\*p<0.001, \*\*\*p<0.0001.

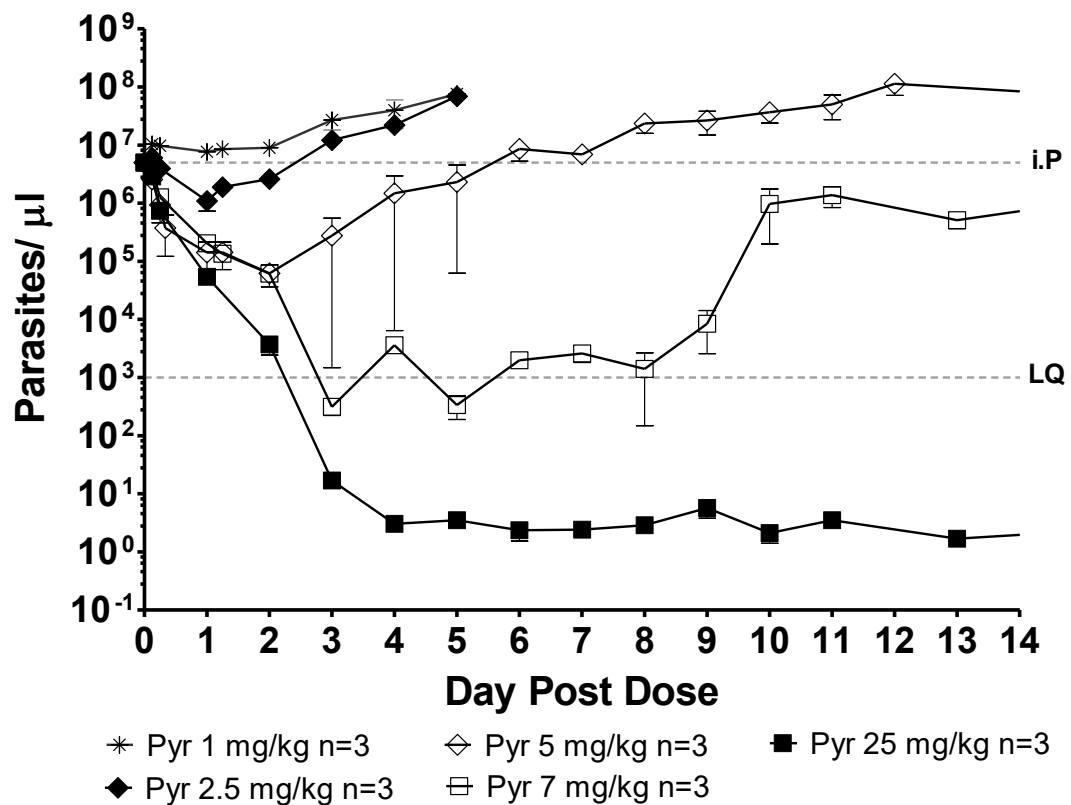


**Table 1 (d): Measure of Parasite Log Reduction and Duration to Return to initial parasitemia. ANOVA (p-value > 0.05 at each time point)**

Dose (mg/kg)	Frequency	6-hr log-reduction	12-hr log-reduction	24-hr log-reduction	48-hr log-reduction	Days to return in initial parasitemia	Mice Cured
10	1			1.1	2.5	4	0
50	1	0.9	1.1	1.1	3.1	7	0
100	1	0.7	1.0	0.9	3.1	11	0
250	1	0.6	0.9	0.7	2.5	Partial Cure	2/3



**Figure 2 (a): Single Oral Dose Killing Profile of Low Dose Pyronaridine in a *P. berghei*ANKA-GFP-Luciferase infection.** Single dose pyronaridine tetraphosphate salt regimen of 1 mg/kg, 2.5 mg/kg, 5 mg/kg, 7 mg/kg and 10 mg/kg was administered orally to Balb/cj female mice (n=3) at time 0-hr which corresponds to ~ 10% parasitemia confirmed by Giemsa blood film counting. Data is represented as mean  $\pm$  SEM.

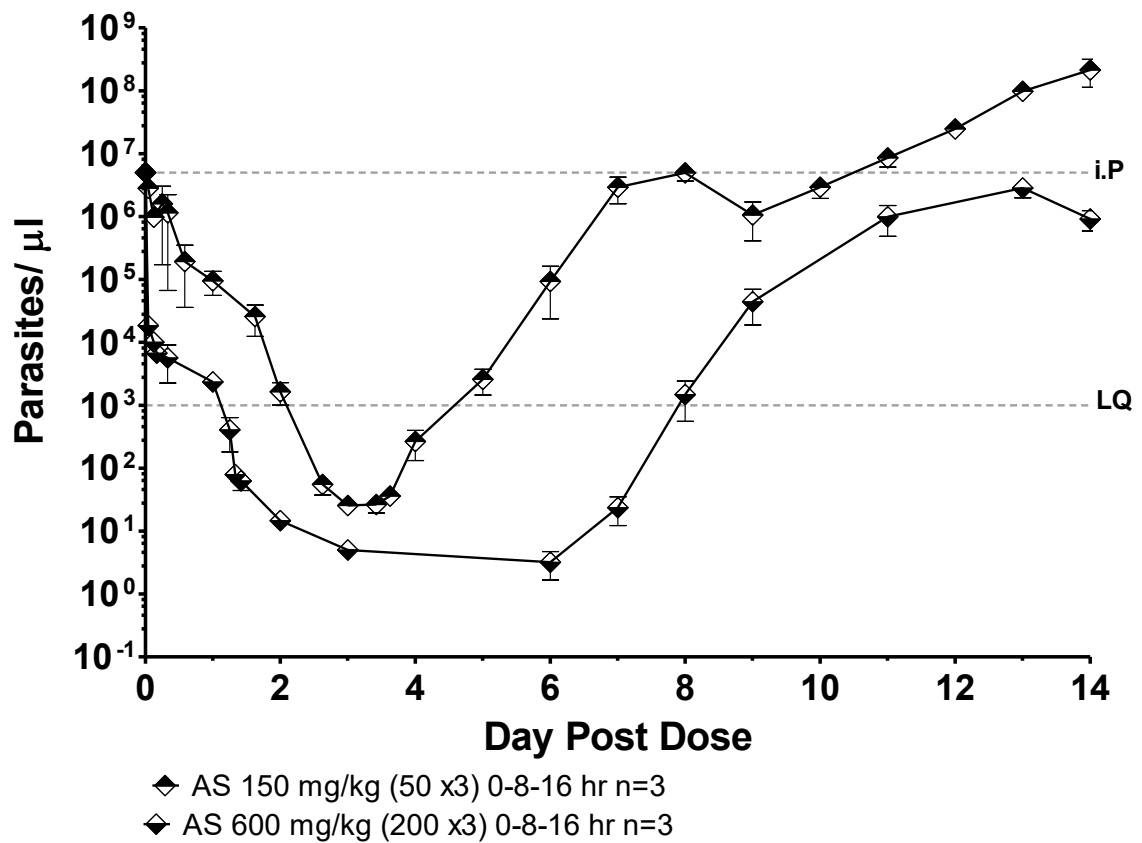


**Table 2 (a): Measure of Parasite Log Reduction and Duration to Return to initial parasitemia. ANOVA \*p-value<0.05**

Dose (mg/kg)	Frequency	6-hr log-reduction	24-hr log-reduction	48-hr log-reduction	Days to return in initial parasitemia	Mice Cured
1	1	- 0.3	- 0.2	- 0.3	0.01	0
2.5	1	0.1	0.7	0.3	2.6	0
5	1	0.7	1.3	1.8	6.8	0
7	1	0.6	1.4	1.9	Partial Cure	2/3
10	1	1.3	3.4	5.1	CURE	3/3

**Figure 2 (b): Killing Profile of Artesunate 3-Dose Regimen in a *P.berghiei*ANKA-GFP-**

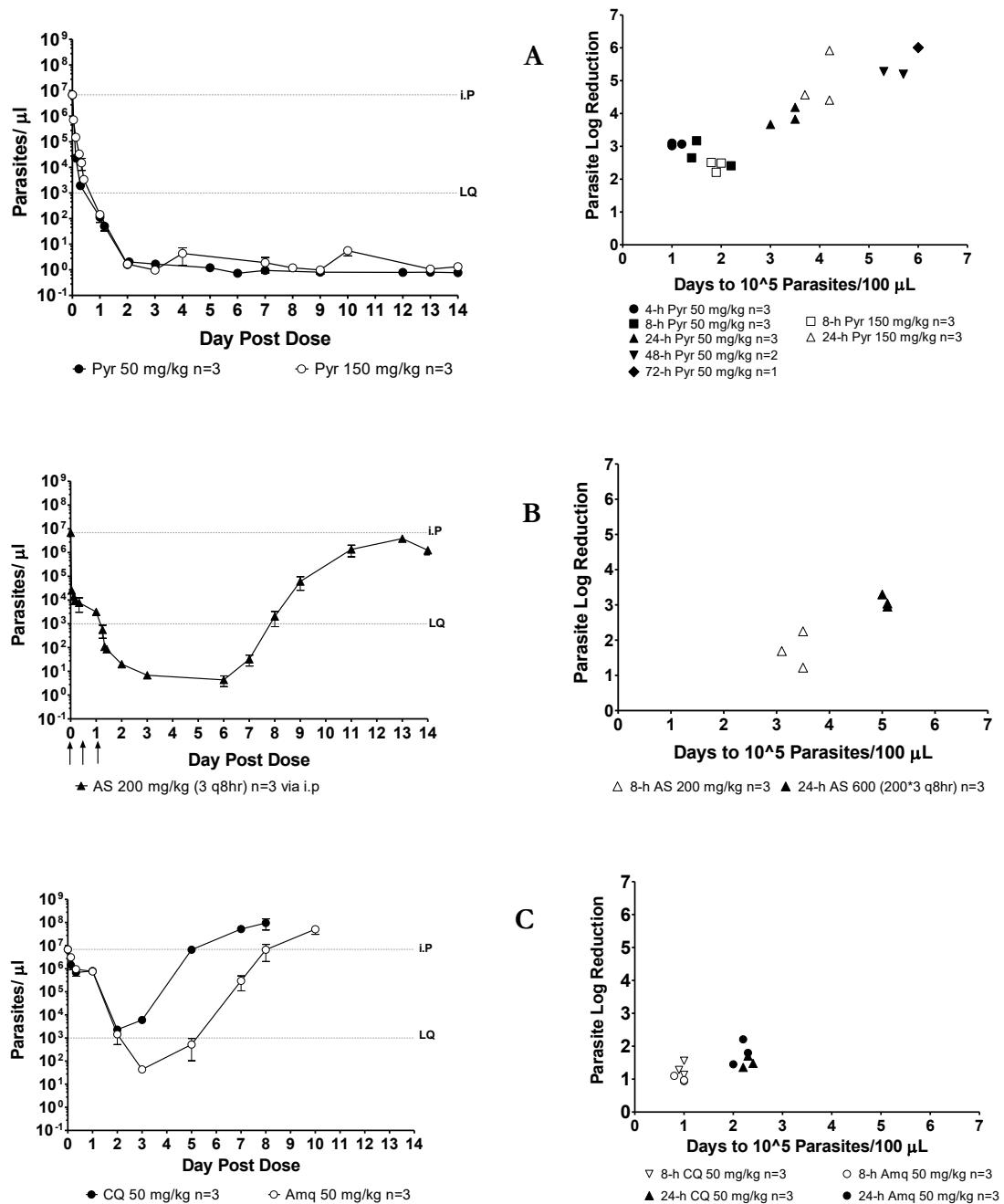
**Luciferase infection.** Three dose regimen of 50 mg/kg and 200 mg/kg were administered intrapetroneally to Balb/cJ female mice (n=3) at time-points 0-hr, 8-hr and 16-hr. Time 0-hr corresponds to ~ 10% parasitemia confirmed by Giemsa blood film counting. Data is represented as mean  $\pm$  SEM. Two-Way ANOVA \*p-value<0.05.



**Table 2 (b): Measure of Parasite Log Reduction and Duration to Return to initial parasitemia. Two-Way ANOVA \*p-value<0.05**

Dose (mg/kg)	Frequency	6-hr log-reduction	24-hr log-reduction	48-hr log-reduction	Days to Return to initial parasitemia	Mice Cured
50	3 (q 8hr)	0.5	1.7	3.5	8	0
200	3 (q 8hr)		3.3	5.5	15	0

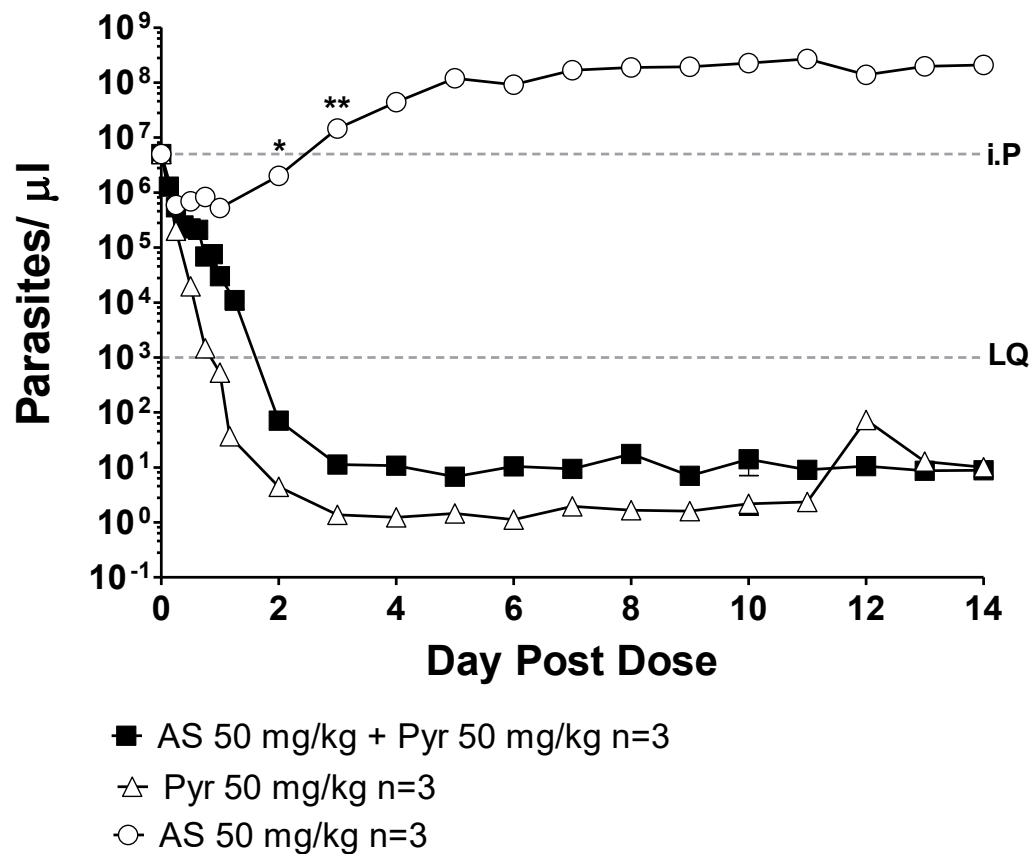
**Figure 3 (a-c): Measure of 8-h and 24-h killing by the long half-life 4-aminoquinolines and short half-life artesunate in an infected blood transfer dilution. (Right) The treated donor kill curve post dosing with drug regimen (mean  $\pm$  SEM). (Left) Scatter plot comparing parasite log reduction to days it takes to reach viable parasites post transfer (represented as individual mouse).**



**Table 3: The 5 microliter quantification Parasite Log Reduction compared to time zero**

Parasite Log reduction at Transfer Injection						
Transfer	8-hr			24-hr		
Drug (mg/kg)	Mouse 1	Mouse 2	Mouse 3	Mouse 1	Mouse 2	Mouse 3
<b>Pyr 50</b>	3.17	2.41	2.65	3.83	3.67	4.19
<b>Pyr 150</b>	2.51	2.21	2.49	5.92	4.41	4.57
<b>AS 200*3 q8hr</b>	2.25	1.22	1.69	3.30	3.04	2.95
<b>CQ 50</b>	1.56	1.28	1.13	1.48	1.70	1.36
<b>Amq 50</b>	0.94	1.10	0.97	1.80	2.21	1.45
Transfer	Pyronaridine 50 mg/kg Parasite Log Reduction					
<b>4-hr</b>	3.02	3.07	3.10			
<b>48-hr</b>	5.20	5.28				
<b>72-hr</b>	6.01					

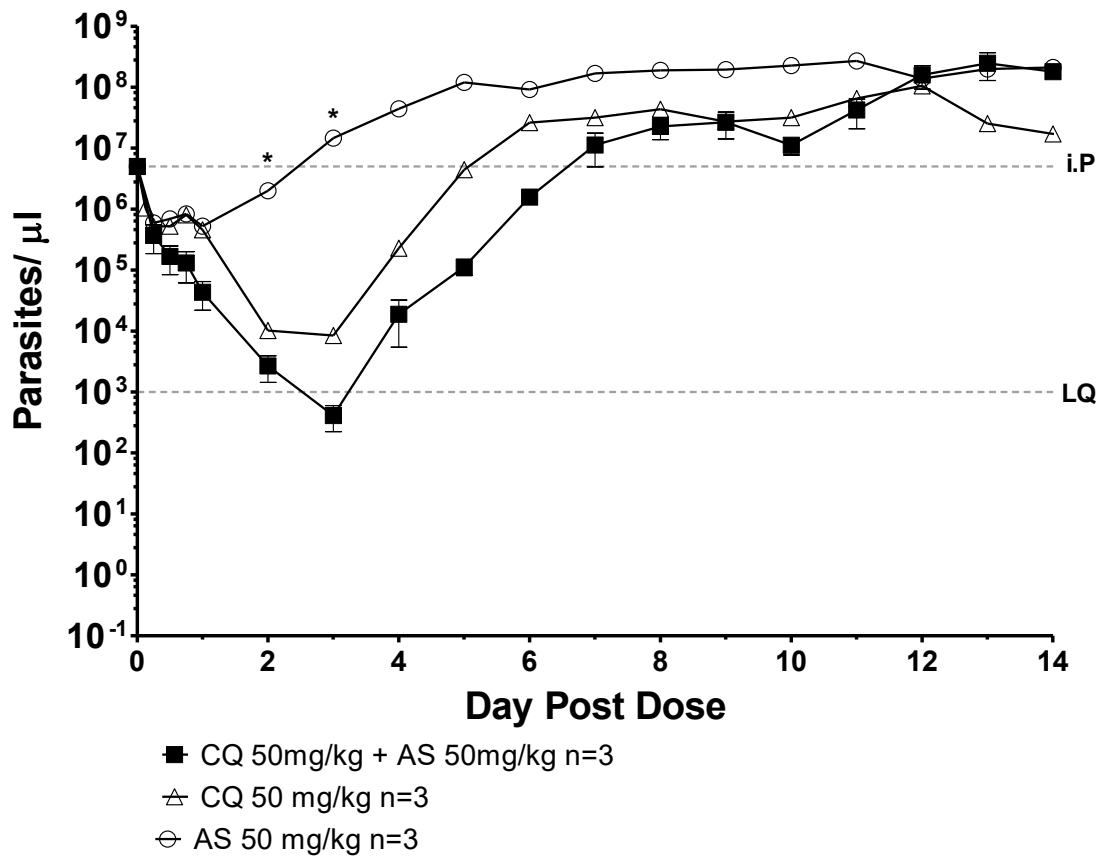
**Figure 4 (a): Effect of Single Dose Combination Therapy with Artesunate and Pyronaridine in a *P.berghei*ANKA-GFP-Luciferase infection.** Single dose regimen of 50 mg/kg pyronaridine tetraphosphate salt and 50 mg/kg artesunate was administered orally to Balb/cJ female mice (n=3) at time-point 0-hr which corresponds to ~ 10% parasitemia confirmed by Giemsa blood film counting. Data is represented as mean  $\pm$  SEM. One-way analysis of variance (ANOVA) \*\*p-value<0.001 \*p-value<0.05



**Table 4 (a): Measure of Parasite Log Reduction and Duration to Return to initial parasitemia**

Drug	Dose (mg/kg)	6-hr log-reduction	12-hr log-reduction	24-hr log-reduction	48-hr log-reduction	Days to Return to initial parasitemia	Mice Cured
Artes	50	0.9	0.9	1.0	0.4	2.5	0
Pyr	50	1.4	2.4	4.0	6.1	CURE	3/3
AS + Pyr	50 + 50	1	1.4	2.2	4.8	CURE	3/3

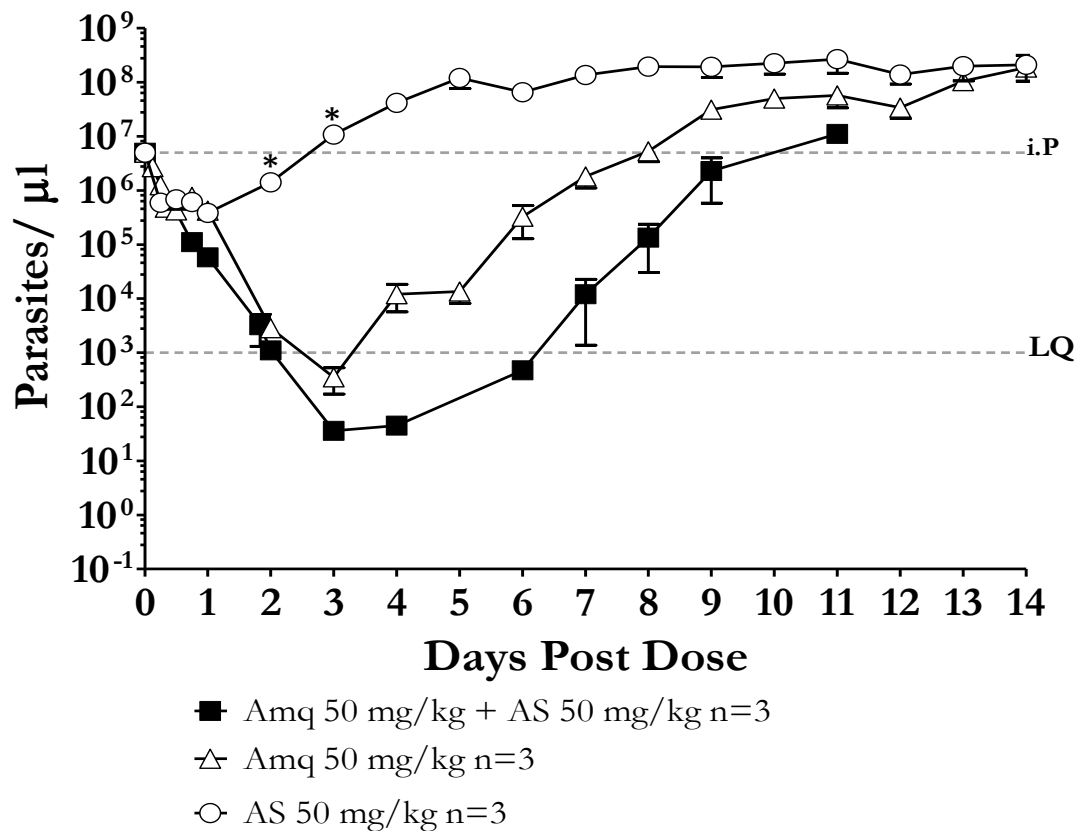
**Figure 4 (b): Effect of Single Dose Combination Therapy with Artesunate and Chloroquine in a *P.berghei*ANKA-GFP-Luciferase infection.** Single dose regimen of 50 mg/kg chloroquine phosphate salt and 50 mg/kg artesunate was administered orally to Balb/cJ female mice (n=3) at time-point 0-hr which corresponds to ~ 10% parasitemia confirmed by Giemsa blood film counting. Data is represented as mean  $\pm$  SEM. One-way analysis of variance (ANOVA) \*p-value <0.05.



**Table 4 (b): Measure of Parasite Log Reduction and Duration to Return to initial parasitemia**

Drug	Dose (mg/kg)	6-hr log-reduction	12-hr log-reduction	24-hr log-reduction	48-hr log-reduction	Days to Return to initial parasitemia	Mice Cured
Artes	50	0.9	0.9	1.0	0.4	2.5	0
CQ	50	0.6	0.9	0.9	2.5	4.8	0
AS + CQ	50 + 50	0.9	1.4	1.9	2.3	6.8	0

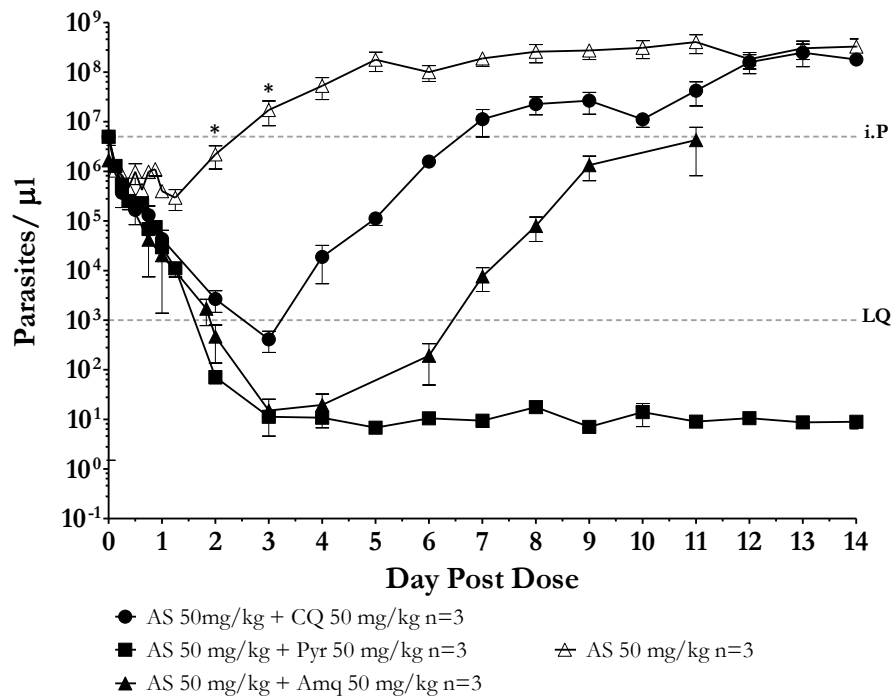
**Figure 4 (c): Effect of Single Dose Combination Therapy with Artesunate and Amodiaquine in a *P.berghei*ANKA-GFP-Luciferase infection.** Single dose regimen of 50 mg/kg (oral) amodiaquine dihydrochloride salt and 50 mg/kg (i.p) artesunate was administered orally to Balb/cJ female mice (n=3) at time-point 0-hr which corresponds to ~ 10% parasitemia confirmed by Giemsa blood film counting. Data is represented as mean  $\pm$  SEM. One-way analysis of variance (ANOVA) \*p-value<0.05.



**Table 4 (c): Measure of Parasite Log Reduction and Duration to Return to initial parasitemia. ANOVA \*p-value<0.05**

Drug	Dose (mg/kg)	6-hr log-reduction	12-hr log-reduction	24-hr log-reduction	48-hr log-reduction	Days to Return to initial parasitemia	Mice Cured
Artes	50	0.9	0.9	1.0	0.4	2.5	0
AMQ	50	0.6	1.1	1.1	3.2	8	0
AS + AMQ	50 + 50			1.9	3.7	10	0

**Figure 4 (d): Effect of Single Dose Combination Therapy with Artesunate and 4-aminoquinolines in a *P. berghei*ANKA-GFP-Luciferase infection.** Single dose regimen of 50 mg/kg 4-aminoquinoline salt and 50 mg/kg artesunate was administered orally/i.p to Balb/cJ female mice (n=3) at time-point 0-hr which corresponds to ~ 10% parasitemia confirmed by Giemsa blood film counting. Data is represented as mean  $\pm$  SEM. One-way analysis of variance (ANOVA) \*p-value<0.05.

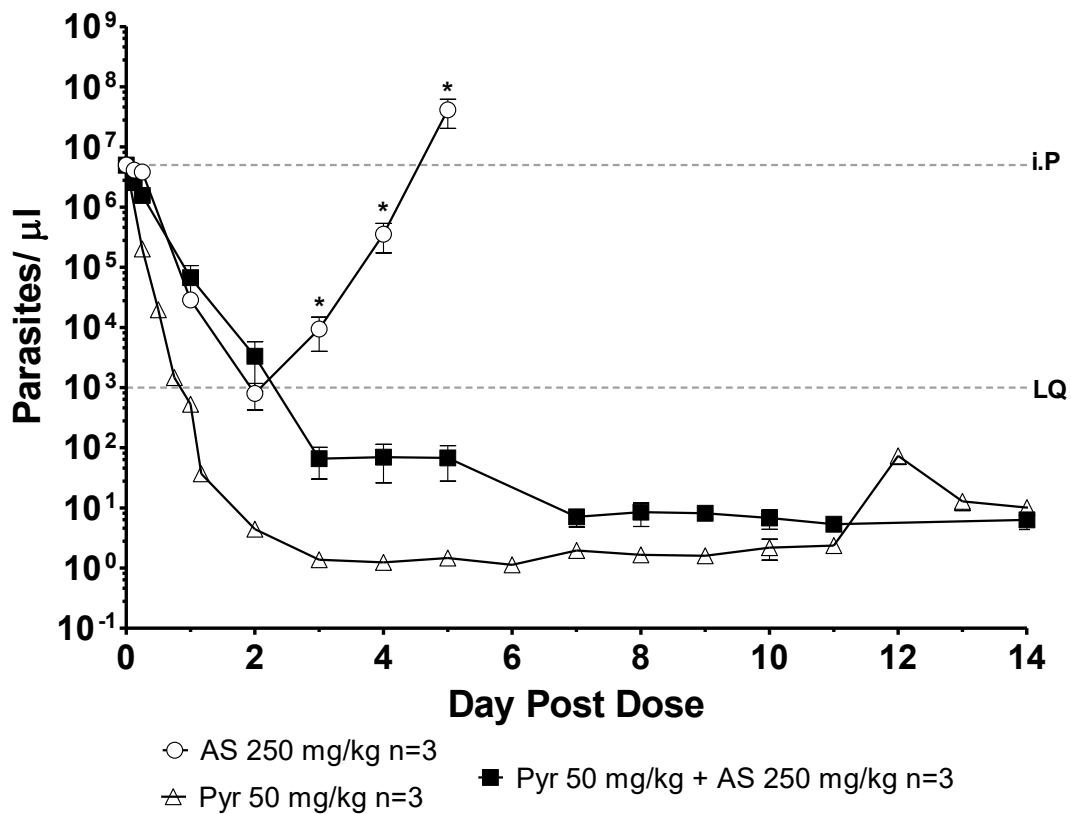


**Table 4 (d): Measure of Parasite Log Reduction and Duration to Return to initial parasitemia. ANOVA \*p<0.05**

Drug	Dose (mg/kg)	6-hr log-reduction	12-hr log-reduction	24-hr log reduction	48-hr log reduction	Days to Return to initial Parasitemia	Mice Cured
AS	50	0.9	0.9	1.0	0.4	2.5	0
AS+Pyr	50+50	1	1.4	2.2	4.8	CURE	3/3
AS+CQ	50+50	0.9	1.4	1.9	2.3	6.8	0
AS+AMQ	50+50			1.9	3.7	10	0



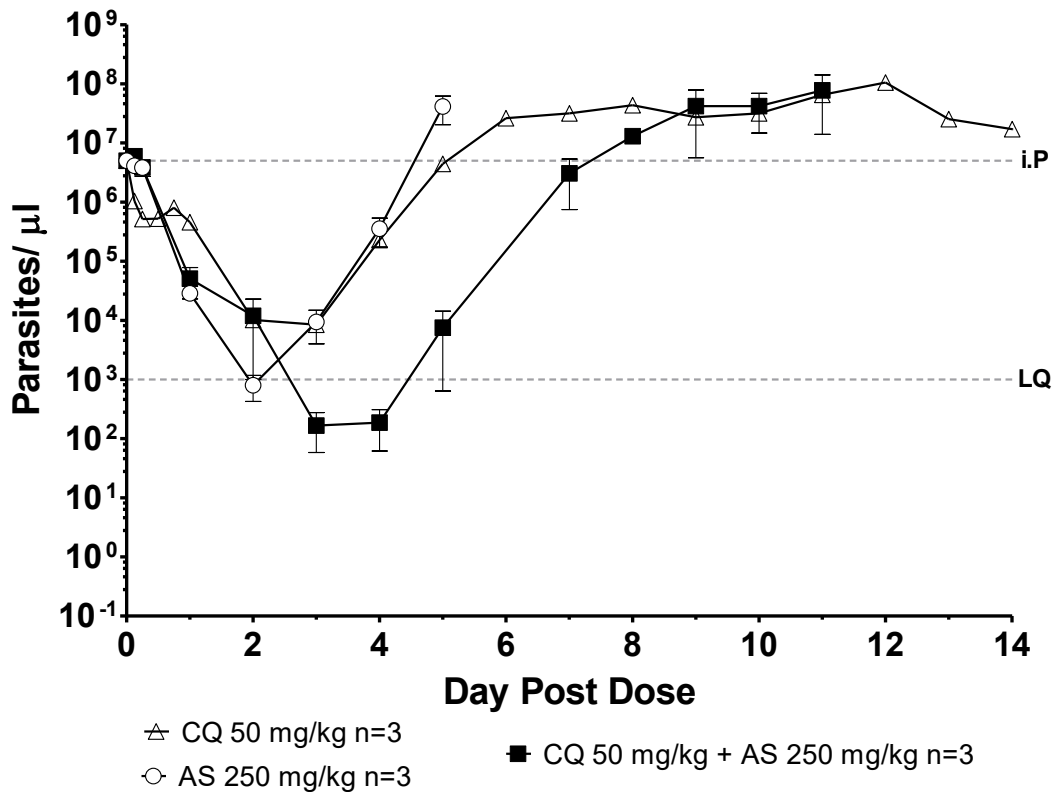
**Figure 5 (a): Effect of Increasing Artesunate Dose in a Single Dose Combination Therapy with Pyronaridine in a *P.berghei*ANKA-GFP-Luciferase infection.** Single dose regimen of 50 mg/kg pyronaridine tetraphosphate salt administered orally and 250 mg/kg artesunate administered via i.p to Balb/cJ female mice (n=3) at time-point 0-hr which corresponds to ~ 10% parasitemia confirmed by Giemsa blood film counting. Data is represented as mean  $\pm$  SEM. One-way analysis of variance (ANOVA) \*p-value<0.05



**Table 5 (a): Measure of Parasite Log Reduction and Duration to Return to initial parasitemia. ANOVA \*p<0.05**

Drug	Dose (mg/kg)	6-hr log-reduction	12-hr log-reduction	24-hr log-reduction	48-hr log-reduction	Days to Return to initial parasitemia	Mice Cured
Artes	250	0.1		2.2	3.8	4.5	0
Pyr	50	1.4	2.4	4.0	6.1	CURE	3/3
AS + Pyr	250 + 50	0.5		1.9	3.2	CURE	3/3

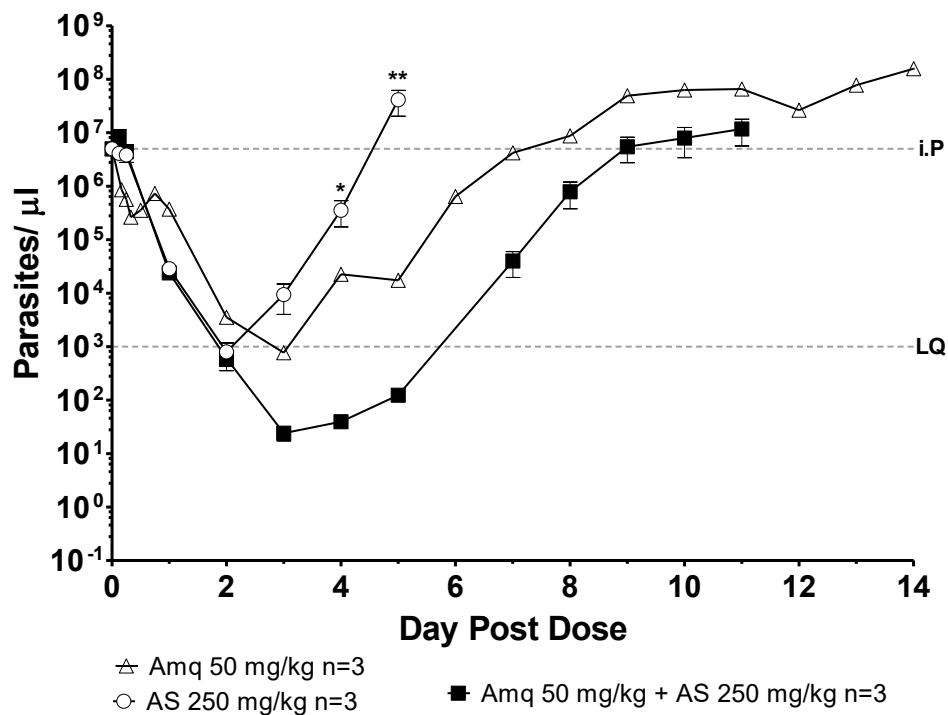
**Figure 5 (b): Effect of Increasing Artesunate Dose in a Single Dose Combination Therapy with Chloroquine in a *P.berghei*ANKA-GFP-Luciferase infection.** Single dose regimen of 50 mg/kg chloroquine phosphate salt administered orally and 250 mg/kg artesunate administered via i.p to Balb/cJ female mice (n=3) at time-point 0-hr which corresponds to ~ 10% parasitemia confirmed by Giemsa blood film counting. Data is represented as mean  $\pm$  SEM.



**Table 5 (b): Measure of Parasite Log Reduction and Duration to Return to initial parasitemia. ANOVA p-value>0.05**

Drug	Dose (mg/kg)	6-hr log-reduction	12-hr log-reduction	24-hr log-reduction	48-hr log-reduction	Days to Return to initial parasitemia	Mice Cured
Artes	250	0.1		2.2	3.8	4.5	0
CQ	50	0.6	0.9	0.9	2.5	4.8	0
AS + CQ	250 + 50	0.1		2.0	2.6	7	0

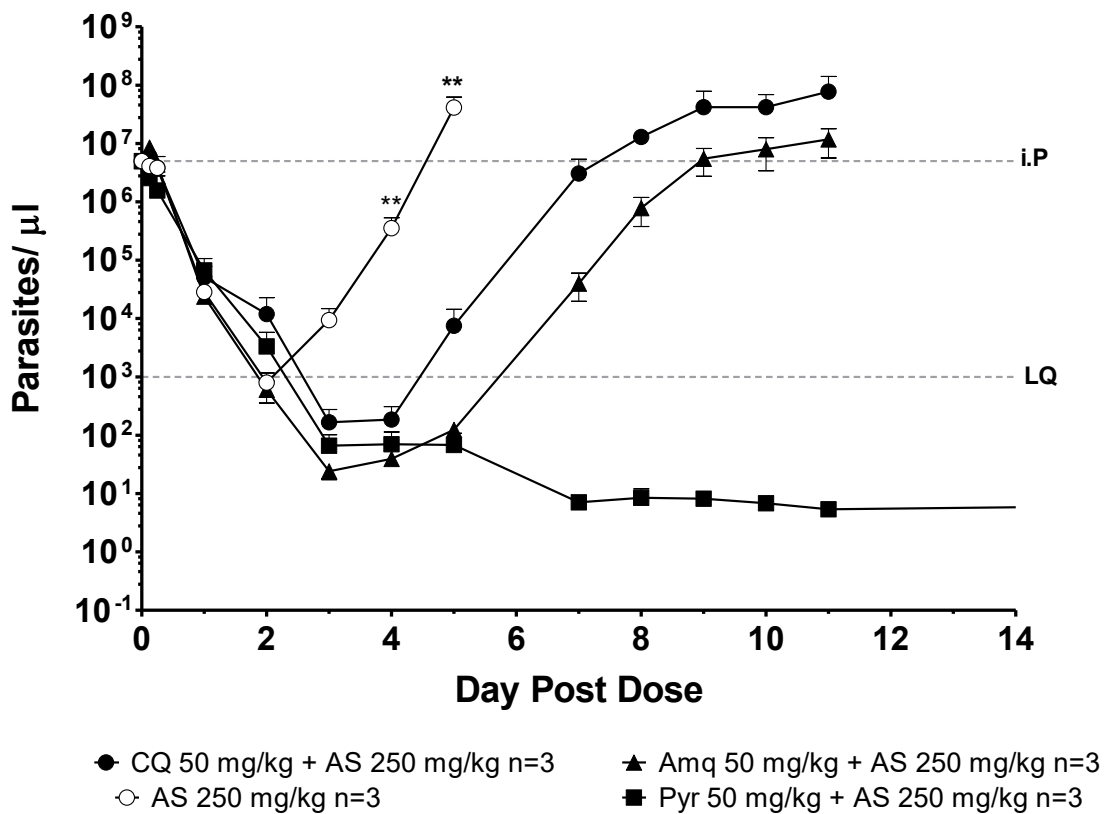
**Figure 5 (c): Effect of Increasing Artesunate Dose in a Single Dose Combination Therapy with Amodiaquine in a *P.berghei*ANKA-GFP-Luciferase infection.** Single dose regimen of 50 mg/kg amodiaquine dihydrochloride salt administered orally and 250 mg/kg artesunate administered via i.p to Balb/cJ female mice (n=3) at time-point 0-hr which corresponds to ~ 10% parasitemia confirmed by Giemsa blood film counting. Data is represented as mean  $\pm$  SEM. One-way analysis of variance (ANOVA) \*p-value<0.05 \*\*p-value<0.001.



**Table 5 (c): Measure of Parasite Log Reduction and Duration to Return to initial parasitemia. ANOVA p-value>0.05**

Drug	Dose (mg/kg)	6-hr log-reduction	12-hr log-reduction	24-hr log-reduction	48-hr log-reduction	Days to Return to initial parasitemia	Mice Cured
Artes	250	0.1		2.2	3.8	4.5	0
AMQ	50	0.9	1.1	1.1	3.1	7	0
AS + AMQ	250 + 50	0.1		2.3	3.9	9	0

**Figure 5 (d): Effect of Increasing Artesunate Dose in a Single Dose Combination Therapy with 4-aminoquinolines in a *P.berghei*ANKA-GFP-Luciferase infection.** Single dose regimen of 50 mg/kg 4-aminoquinoline salt administered orally and 250 mg/kg artesunate administered via i.p to Balb/cJ female mice (n=3) at time-point 0-hr which corresponds to ~ 10% parasitemia confirmed by Giemsa blood film counting. Data is represented as mean  $\pm$  SEM. ANOVA \*\*p<0.001.



**Table 5 (d): Measure of Parasite Log Reduction and Duration to Return to initial parasitemia.** ANOVA p-value>0.05 for each time point.

Drug	Dose (mg/kg)	6-hr log-reduction	24-hr log-reduction	48-hr log-reduction	Days to Return to initial parasitemia	Mice Cured
AS	250	0.1	2.2	3.8	4.5	0
AS + Pyr	250 + 50	0.5	1.9	3.2	CURE	3/3
AS + CQ	250 + 50	0.1	2.0	2.6	7	0
AS + AMQ	250 + 50	0.1	2.3	3.9	9	0

## CHAPTER 4 - DISCUSSION

These studies assessed the *in vivo* pharmacodynamic properties of artesunate and the 4-aminoquinolines – pyronaridine, amodiaquine, chloroquine, given in a single dose regimen and also evaluated the interaction between artesunate and each of the 4-aminoquinolines in a combination therapy. The findings clearly demonstrate that pyronaridine is the most potent of all the antimalarial agents tested in this model. Single oral dose pyronaridine  $\geq 10$  mg/kg is more potent than both artesunate administered at single and 3-dose regimen. Artesunate exhibits dose-dependent killing when administered at both the single and multi-dose regimens- the higher the dose the longer the duration to return to initial parasitemia. Initial dose-independent killing was observed with chloroquine and amodiaquine, however there is a similar plateau phase of undetected parasite killing observed between 6 hours and 24 hours post initial dose. Dose-independent killing and complete cure was achieved with pyronaridine given at single dose  $\geq 10$  mg/kg. However, partial cure was achieved with low dose 7 mg/kg (two-thirds mice cured). Single dose amodiaquine at 250 mg/kg was curative (two-thirds mice cured). However, no cure was achieved with chloroquine and artesunate in this model.

We were interested in the viable parasites at 8 and 24 hours after dosing with the long half-life quinolines. We devised a transfer of washed infected erythrocytes to measure viability and to greatly reduce quinoline concentrations. Following the infected blood transfer experiment, we measured the duration to reach benchmark parasites per microL. A linearity correlation was observed with pyronaridine 50 mg/kg (the higher the parasite log reduction with more time after dosing, the longer the days it took to reach viable parasites). At 24 hours transfer, high dose artesunate took nearly the same amount of days to reach viable parasites as single dose pyronaridine 50 mg/kg at 48-hour transfer. Chloroquine and amodiaquine at both 50 mg/kg single oral dose achieved nearly the same parasite log reduction at both 8 and 24 hours post dosing in the treated donor mice and the

duration to reaching viable parasites post transfer was not that different, about 1 and 2 days post 8 and 24 hours transfers respectively.

Artesunate is short-lived yet acts very rapidly in parasite clearance with nearly 4 log kill eliminating the immature ring stage parasites, however the 4-aminoquinolines are long-acting and act only by killing mature parasites. Therefore, combination of these two different classes with different mechanisms of action will lower risk of drug resistance and recrudescence. (Guatam et al., 2009; Li & Weina 2010). To evaluate this, the effect of single dose dynamic killing in combination therapy with artesunate and 4-aminoquinolines was assessed. For the combination therapy, the mice groups received a single dose of artesunate and 4-aminoquinoline at the same time administered intraperitoneally and orally respectively. Chloroquine or amodiaquine 50 mg/kg when given in combination with artesunate has an additive interaction and increased parasite log reduction at 24 hours post dosing compared to CQ or AS given alone. Parasite log reduction was increased to almost that of artesunate given alone at 50 mg/kg or 250 mg/kg, clearly shows that pharmacological effect of chloroquine or amodiaquine was enhanced by artesunate. Our data findings also showed a slightly weak antagonistic interaction between artesunate and pyronaridine combination though it achieved cure. Overall, in the second model increasing artesunate dose by 5 fold showed increased killing effect for combinations with chloroquine and amodiaquine, increased duration to return to initial parasitemia for amodiaquine-artesunate combination and nearly the same duration for chloroquine-artesunate combination.

Pyronaridine has been used in China for malaria treatment since 1970 when it was initially discovered. Pyronaridine is readily absorbed and distributed to the tissues following oral administration and it is well tolerated and effectively eliminated in the urine. (Park SH and Pradeep K 2010). Studies utilizing mouse models have strongly and clearly indicated that pyronaridine has high efficacy against the asexual blood stages of the *Plasmodium* parasite. In other studies, it was demonstrated that pyronaridine exhibits longer duration schizontocidal activity than chloroquine

while other studies utilizing *P. berghei* model have suggested that pyronaridine in combination with artesunate allows for lower dose of pyronaridine to be used than when given as monotherapy. *In vivo* clinical studies have shown pyronaridine to be very potent against both *P. falciparum* parasites and chloroquine-resistant strains. However, there has been conflicting data that has been published regarding the *in vitro* activity of pyronaridine in combination with other antimalarial agents. Studies have reported weak antagonism, synergistic and additive interactions when pyronaridine is combined with artemisinins, additive effect when combined with other 4-aminoquinolines and synergy when combined with primaquine. All these conflicting differences might be due to different methods and *in vivo* analyses used. (Chang et al., 1992; Basco et al., 1999; Shao et al., 1984; Vivas et al., 2008; Shao 1990; Ruscoe et al., 1998; Shao et al., 1992).

Several studies have assessed the pharmacodynamics drug-drug interactions *in vitro* and *in vivo*. Particularly for artesunate-pyronaridine combination therapies, there has been conflicting results where as some findings showcase antagonistic interactions and others find a synergistic or additive interaction. For instance, a group (Vivas et al., 2008) evaluated the efficacy of pyronaridine and artesunate, and their studies showed slight antagonism with *P. falciparum* but the *in vivo* studies with rodent *P. berghei* found increased activity that suggested additive or synergistic interactions. However it is suggested that the combination should be further explored in clinical settings.

A study by a group (Gupta et al., 2002b) also assessed pharmacodynamic drug-drug interactions in combination of artemisinin with 4-aminoquinolines (amodiaquine, pyronaridine and chloroquine) tested in three different strains of *P. falciparum* – one chloroquine-resistant and two chloroquine-sensitive strains. They found a synergistic pharmacodynamics interaction between artemisinin-amodiaquine and artemisinin-pyronaridine combinations. However, for the chloroquine-artemisinin combination, their studies found additive pharmacodynamics interaction. Currently, artesunate-amodiaquine is one of the first-line recommended ACTs while artesunate-pyronaridine (Pyramax®) is currently being investigated on though it's already being used in some of the malaria

endemic areas such as Kenya but it has not yet been recommended by the WHO as one of the ACTs for malaria treatment.

Resistance to pyronaridine has been shown to appear slowly in the field however in combination with artesunate the drug resistance is slowed. Clinical studies that have utilized pyronaridine monotherapy or combination therapy to treat patients infected with blood stage vivax and falciparum malaria have reported pyronaridine to be efficacious for the treatment of the blood stage infections. Currently pyronaridine-artesunate (3:1) fixed dose synergistic combination (Pyramax®) is used for the treatment of uncomplicated falciparum malaria and blood stage vivax malaria (Croft et al., 2012).

Artemisinin combination therapy is currently considered first line treatment of uncomplicated and severe malaria. Drug combination is considered one of the effective ways to counteract drug resistance. Therefore, ideal drug partner to artemisinins should exert synergistic or additive schizontocidal action. Even though some studies have shown additive interactions between pyronaridine and other 4-aminoquinolines, combination of the 4-aminoquinolines alone may not be useful in malaria treatment due to the wide spread resistance to these drugs in the malaria endemic areas.

Studying antimalarial interactions between or among drugs used to treat malaria is very paramount. Pharmacodynamic interactions do exist between antimalarial drugs as has been shown by the *in vitro* as well as *in vivo* study models and clinical trials that have assessed additive, synergistic, antagonistic combination therapies. However, there's still more studies need to be done to clearly elucidate these controversial findings, which might be methodology biased.



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# CURRICULUM VITAE

## Winter A. Okoth

Johns Hopkins University Bloomberg School of Public Health

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### EDUCATION

**Johns Hopkins University Bloomberg School of Public Health** *Baltimore, MD, USA*

Masters of Science (ScM) in Molecular Microbiology and Immunology

2015 - 2017

Thesis title: **“Single Dose Killing Pharmacodynamics of Antimalarial Drugs in a Luciferase Murine Malaria Model”**

Thesis Advisor: Dr. David Sullivan

**Thomas More College**

*Crestview Hills, KY, USA*

Bachelor of Arts (BA) – Biology Major

2008 - 2012

Concentration – Cellular and Molecular

Associates of Arts (AA) – Chemistry

Honors: Cum Laude

**St. Gabriel’s Gagra Secondary School**

*Kisumu, Kenya, Africa*

Kenya Certificate of Secondary Education (KCSE)

2002 - 2005

### HONORS, AWARDS, AND FELLOWSHIPS

**Honors and Awards Recipient**

*Johns Hopkins University Bloomberg School of Public Health*

2017

**Emergent Biosolutions Fellowship**

*Emergent Biosolutions*

2016

**Master’s Tuition Scholarship**

*Johns Hopkins University Bloomberg School of Public Health*

2016 – 2017

**Masters Tuition Scholarship**

*Benevolent Well-wishers*

2015 – 2017

**AAUW International Fellowship**

*Association of American University Women Organization (AAUW)*

2015 - 2016

**Full Time Academic & Need-Based Scholarship**

*Thomas More College*

2008 – 2012

**Academic Dean's List and Honors***Thomas More College* 2008 – 2012**Resident Assistant Room & Board Grant***Thomas More College* 2009 – 2012**Delta Epsilon Sigma Honors Society - Inducted Member***Thomas More College* 2012**Honors Convocation - Academic Achievement Certificate***Thomas More College* 2012**Student Research Forum Certificate***Thomas More College* 2012**Tri-beta Biological Honors Society – Inducted Member***Thomas More College* 2008 – 2012**Honors Convocation - Academic Achievement and Student Services Award***Thomas More College* 2011**US-Mexico Border Studies Service Learning Global Travel Grant Award***Thomas More College* 2011**Summer Undergraduate Research Fellowship***Mayo Clinic College of Medicine, Allergic Diseases Research Laboratory, Immunology Department* 2011**Summer Undergraduate Research Fellowship***Mayo Clinic College of Medicine, Allergic Diseases Research Laboratory, Immunology Department* 2010**RESEARCH EXPERIENCE****Johns Hopkins University Bloomberg School of Public Health****Baltimore, MD, USA**

Department of Molecular Microbiology and Immunology

*Nov. 2015 – May 2017*

Masters Student in the Laboratory of Dr. David Sullivan

- Helped pioneer pharmacodynamics research in the laboratory of David Sullivan with key interest on artesunate compared to 4-aminoquinolines. Thesis outcome is a novel ranking of malaria drugs by parasitocidal properties rather than inhibition properties
- Investigated on the single dose killing pharmacodynamics properties of antimalarial drugs namely artesunate compared to 4-aminoquinolines – chloroquine, pyronaridine and amodiaquine using luciferase-based murine malaria model under supervision of principal investigator. Malaria metrics measured include: drug killing rate, length of undetectable parasitemia, timing of recrudescence parasites and measure of cure.
- Designed experiments through guidance and active consultations with the principal investigator.
- Independently conducted experiments, collected and analyzed data.
- Actively participated and gave both oral and poster presentations at lab meetings, forums and conferences

**Johns Hopkins Malaria Research Institute****Baltimore, MD, USA**

Part-Time Research Assistant in the Laboratory of Dr. Conor McMeniman

*Nov. 2015 – May 2017*

- Actively performed rearing and maintenance of the both transgenic and wildtype mosquito stocks in the insectary for McMeniman and Potter's laboratories.
- Maintained the mosquito cell lines and drosophila fly food
- Created the inventory of mosquito strains, and helped with other causal lab duties as needed

**Kenya Medical Research Institute (KEMRI)****Nairobi, Kenya**

Center for Microbiology Department

*Oct. 2014 – Mar. 2015*

Volunteer Clinical Research Intern

- Actively participated in the clinical research project on *Staphylococcus spp.* led by Dr. Obanda.
- Connected with some of the scientific researchers in Kenya
- Shared research expertise as well as mentoring the undergraduate students on attachment

**Mayo Clinic College of Medicine**

***Rochester, MN, USA***

Department of Biochemistry & Molecular Biology

*Jun. 2012 – Dec. 2013*

Research Technologist

- Actively engaged in the protein misfolding and cellular cytotoxicity research projects
- Set up and performed routine and established lab procedures in support of lab goals
- Successfully trained on and performed cell/tissue culture assays; expressed, extracted and purified Amyloidogenic light chain mutant proteins.
- Collected, summarized and analyzed data by performing basic statistical data analyses, and presented at laboratory meetings.
- Achieved first scientific publication and co-authorship in the Journal of Liposome Research.
  - **Key Role:** Successfully expressed, extracted and purified the Amyloidogenic light chain mutant proteins used in the experiments.

**Mayo Clinic College of Medicine**

***Rochester, MN, USA***

Department of Immunology

*May – Aug. 2010 & 2011*

Summer Undergraduate Research Fellow

- Participated in two extensive summer research projects at the Allergic Diseases Research Lab.
- Investigated on the dynamics of a novel subset of innate lymphocytes in the lungs in response to various cytokines and their role in allergic asthma. Trained in laboratory and research specific methods, techniques and relevant literature.
- Designed and conducted experiments, under guidance of principal investigator and senior research scientist. Gained more laboratory skills regarding various protocols used such as running ELISAs, tissue culture and flow cytometry, performed the lung surgeries on mouse research models. Collected and interpreted data; made novel observations; drew sound scientific conclusions based on the data analysis. Gave oral and poster presentation

## PUBLICATIONS

### Articles

1. Truran S, Weissig V, Ramirez-Alvarado M, Franco DA, Burciu C, Georges J, Murarka S, **Okoth WA**, Schwab S, Hari, P, Migrino R. “Nanoliposomes protect against AL amyloid light chain protein-induced endothelial injury”. Journal of Liposome Res. (2013). Mar; 24(1):69-73. doi: 10.3109/08982104.2013.838258. Epub 2013 Nov 15.
2. **Okoth WA** and Sullivan DJ. “Single dose superior pharmacodynamics of pyronaridine compared to artesunate, chloroquine and amodiaquine in a high density murine malaria model” First author paper indicating that the new investigational drug pyronaridine is more potent than artesunate. Currently work-in-progress with tentative submission to the journal by early summer 2017.

### Blog

1. “Career Advice and Tips for College Students” - Contributing Author at Project Eve blog – authored article: <http://www.projecteve.com/career-advice-for-college-students> Oct. 2013

## SCIENTIFIC PRESENTATIONS

### Conference / Poster/ Oral Presentations

1. Masters Thesis Defense Presentation – Oral  
MMI Department, Johns Hopkins Bloomberg School of Public Health, Baltimore MD 2017
2. American Society of Tropical Medicine & Hygiene ASTMH - Poster  
65<sup>th</sup> Annual Meeting in Atlanta, Georgia 2016
3. Future of Malaria Research, 2<sup>nd</sup> Annual Young Scientist Meeting - Poster  
Johns Hopkins Malaria Research Institute, Rockville MD 2016
4. Molecular Microbiology & Immunology Departmental Retreat - Poster  
Johns Hopkins University Bloomberg School of Public Health, Baltimore MD 2016
5. MMI Department Research Forum – Oral  
Johns Hopkins University Bloomberg School of Public Health 2016
6. World Malaria Day: ICEMRS Conference – Poster  
Johns Hopkins University Bloomberg School of Public Health 2016
7. Student Research Forum – Poster  
Thomas More College 2012
8. Summer Undergraduate Research Fellowship – Oral and Poster  
Immunology Department, Mayo Clinic College of Medicine, Rochester MN 2010 & 2011
9. Kentucky Academy of Science – Poster  
Western Kentucky University, Kentucky 2010

## PROFESSIONAL MEMBERSHIPS

1. American Society of Tropical Medicine & Hygiene (ASTMH) 2016 – Present
2. American Association of University Women (AAUW) 2015 – Present
3. Harambe Entrepreneur Alliance, Associate Class of 2013 2013 – Present
4. Project Eve Blog, Contributing Author 2013 – Present
5. Association for Women in Science (AWIS) 2013 – 2015
6. Diaspora African Women Network (DAWN) 2013 – 2014
7. Thomas More College
  - a. Biology & Chemistry Clubs
  - b. International Student Society
  - c. Business Society, Habitat for Humanity
  - d. African American Student Association, member and secretary
  - e. English Club, Words Journal Co-editor and author 2009-2010

## LEADERSHIP AND PUBLIC SERVICE

1. **Pamoja Kenya Mentorship Alliance (P.A.K.E.M.A) Organization** *2012 - Present*
  - a. Founded the above organization in 2012, a 501c3 non-profit organization based in both in KY, USA and Kenya. As an alliance of Kenyans & Global elites extending networking and mentorship resources to school children and youths situated in the underprivileged areas of Kenya & beyond by promoting leadership, academic growth, skills development and empowerment of the next generation of young leaders.
  - b. Committed volunteer mentor and executive director overseeing the complete operation of the organization in accordance with the directions established in the PAKEMA strategic plan.
  - c. Engaged in leadership, development and growth of PAKEMA programs.
2. Social Media Coordinator, Molecular Microbiology & Immuno. Dept., JHSPH *2015-2016*
3. 2015 National Innovation Challenge for High School Students, Mentor *2015*
  - a. Mentored young innovators of organic soap. The challenge was held at the Center for Mathematics, Science, and Technology Education in Africa (CEMASTEA), hosted by the Global Minimum & Innovate Kenya Organizations.
4. UN Women Initiative – HeforShe Campaign Kenya Team – Volunteer *11/2014 – 3/2015*
5. International Women’s Day Siaya County Commemoration, Speaker *Mar. 2015*
6. Ashoka Change-Makers Africa Future Forward Bootcamp Nairobi, Participant *Feb. 2015*
7. Take Lead Women Leadership Program, Participant *Oct. 2013*
8. Harambe Entrepreneur Alliance, Associate Class of 2013 *Apr. 2013*
  - a. Attended and presented at the Harambe Bretton Woods Symposium (HBWS) held at Babson College, Harvard Kennedy School and Mt. Washington Hotel Bretton Woods NH, USA
9. Thomas More College *2008 – 2012*
  - a. Distinguished Alumnae League Leadership & Mentorship Roundtable Participant:
  - b. Resident Assistant, Biology & Chemistry Tutor,
  - c. Student Ambassador Biology, Admission Office
  - d. International Student Society President & Volunteer Science Fair Judge at NACKES
  - e. Volunteer Habitat for Humanity & US-Mexico Border service learning studies
  - f. Orientation Team, assistant director and group leader

## INTERVIEWS, FEATURES AND INVITATIONS

- Johns Hopkins University STAR students – Alumni Council Leadership Summit *Oct. 2016*
- L’Oréal Women in Science Awards Dinner, AAUW Washington DC *Oct. 2016*
- President Obama’s Young African Leaders Initiative Entrepreneurship Awards *Aug. 2016*  
Dinner hosted by United States African Development Fund (USADF)
- Johns Hopkins Bloomberg School of Public Health Summer Magazine Feature *June 2016*  
<http://magazine.jhsph.edu/2016/summer/features/all-that-buzz/>
- Standard Newspaper Kenya, Profile Feature *May 2016*
- Center for Microbiology Dept., KEMRI Mentorship Workshop, Speaker *Apr. 2015*
- Ebru Africa TV – Africa This Morning Table Talk on Youth Empowerment *Apr. 2015*
- German Embassy Nairobi, Consultative meeting *Mar. 2015*  
Youth empowerment and how to unleash the potential of youth cooperation between Kenya and Germany.
- Africa Creates – Online Radio and Ramogi FM News Hour, Nairobi Kenya *Feb. 2015*
- Kenyan Woman Publication – Profile Feature *Nov. 2014*



- London-based Zars Media Profile Feature on “Working Women” Oct. 2013
- Chicago, Jane Thang Productions Feature “Classy and Empowered” Project Sept. 2013
- Invitation to give talks  
 American Association of University Women (AAUW) – Baltimore & MD Chapters *2016*  
 JKUAT University Biochemical Association mentorship session Nairobi Kenya, *2015*  
 Rotary International, Kentucky chapter *2009, 2014*  
 St. Henry Elementary Youth group program *2014*  
 Northern Kentucky University Biological Honors Society *2014*  
 Thomas More College *2009-2012*

## LANGUAGE SKILLS

English: Fluent as Native Speaker | Swahili: Native Speaker | Luo: Native Speaker | Spanish: Conversational.